Anal. Calcd for $C_{19}H_{22}N_4O$: C, 70.78; H, 6.88; N, 17.38. Found: C, 70.60; H, 6.80; N, 17.30.

The **monohydrochloride** of Ic, prepared by addition of 1 equiv of dilute HCl to an ethanolic solution of Ic followed by evaporation to dryness under reduced pressure, was recrystallized from MeOH–EtOAc; pale yellow crystals: mp 232–234°; $\lambda_{max}^{EtOH-H_2O}$ 249, 345 mμ (ε 20,300, 11,800).

Anal. Calcd for $C_{19}H_{22}N_4O \cdot HCl$: C, 63.59; H, 6.46; N, 15.61; Cl^-, 9.88. Found: C, 63.80; H, 6.30; N, 15.30; Cl^-, 9.70.

6-Methoxy-10-hydroxy-2,9-diazaanthracene (IIc, 6-Methoxypyrido[3,4-*b*]quinolin-5(10H)-one).—A mixture of 2.5 g (0.01 mole) of 3-(*p*-anisidino)isonicotinic acid, 40 g of polyphosphoric acid, and 2 ml of $POCl_3$ was heated on a steam bath, with manual stirring, for 7 hr until the evolution of HCl could no longer be detected. The syrup was added to 200 ml of ice–H_2O and the resulting solution was made basic with NH_4OH. The yellow solid was collected, washed with H_2O, and dried in air. On recrystallization from MeOH, 2.0 g (87%) of IIc was obtained as golden yellow leaflets: mp 333–335° dec (uncor); λ_{max}^{EtOH} 242, 279, 309, 322, 408, 425 mμ (ε 28,000, 40,000, 3600, 2500, 8800, 8100); $\lambda_{max}^{pH\ 1}$ 294, 458 mμ (ε 32,000, 6800).

Anal. Calcd for $C_{13}H_{10}N_2O_2$: C, 69.02; H, 4.46; N, 12.38. Found: C, 69.29; H, 4.46; N, 12.50.

6-Methoxy-10-chloro-2,9-diazaanthracene (IId).—A mixture of 2.5 g (0.01 mole) of IIc and 60 ml of $POCl_3$ was heated under reflux for 15 hr. After removal of the excess $POCl_3$ (reduced pressure), the mixture was poured onto 200 g of crushed ice and made basic with NH_4OH. The resulting solid product was collected, washed (H_2O), and dried in air. It was recrystallized (Me_2CO) to furnish 2.25 g (83%) of IId as yellow needles: mp 187–188°; λ_{max}^{EtOH} 229, 237, 258, 362 mμ (ε 33,000, 32,000, 74,000, 11,000).

Anal. Calcd for $C_{13}H_9ClN_2O$: C, 63.81; H, 3.71; N, 11.45. Found: C, 63.64; H, 3.61; N, 11.48.

6-Methoxy-10-(3-diethylaminomethyl-4-hydroxyanilino)-2,9-diazaanthracene (IIb).—2-Diethylaminomethyl-4-acetamidophenol (3.07 g, 0.013 mole) was boiled with 20 ml of 20% HCl for 3 hr. The solution was allowed to cool and was neutralized with 10% NaOH to pH 6. Compound IId (3.18 g, 0.013 mole) was then introduced. The mixture was heated on a steam bath for 6 hr, cooled, diluted with 50 ml of H_2O, and made basic with NH_4OH. The precipitate was collected, washed (H_2O), and allowed to dry in air. On recrystallization (EtOAc) there was obtained 3.80 g (73%) of IIb as orange-red crystals, mp 189–193°. Further recrystallization raised the melting point to 191–193°; λ_{max}^{EtOH} 252, 290, 445 mμ (ε 41,000, 24,000, 10,000).

Anal. Calcd for $C_{24}H_{26}N_4O_2$: C, 71.62; H, 6.51; N, 13.92. Found: C, 71.53; H, 6.47; N, 14.02.

Attempted Preparation of IIa. Isolation of 6-Methoxy-2,9-diazaanthracene (IIe).—A mixture of 4.90 g (0.02 mole) of IId and 17 ml of 1-diethylamino-4-aminopentane was heated at 145–150° for 5 hr (N_2 atmosphere). The mixture was diluted with 100 ml of H_2O, made basic with NH_4OH, and filtered to remove the precipitated IIc (0.55 g). The filtrate was extracted repeatedly with $CHCl_3$ (total, 250 ml), and the combined extracts were washed (H_2O), dried ($MgSO_4$), and evaporated at atmospheric pressure. After excess dialkylaminoalkylamine was removed (rotary evaporator, steam bath), the tarry substance, which did not solidify on standing, was collected and subjected to short-path distillation (burner, heat source). There was obtained at 220–230° (1 mm) 0.85 g of a yellow solid, mp 136–138°. Recrystallization from EtOAc gave light yellow crystals, mp 139.5–140.5°. Its spectrum ($CDCl_3$) showed aromatic proton signals at τ 3.46 (1 H, doublet), 2.83 (2 H, 2 doublets), 2.27 (1 H, doublet), 2.18 (1 H, singlet), 1.84 (1 H, doublet), and 0.72 (1 H, singlet) and the methoxy singlet at 6.3 (3 H); infrared, 6.13, 6.21, 6.34, 6.68, 7.16, 7.9, 8.5, 8.9, 9.8, 11.0, 12.0, and 12.4 μ; λ_{max}^{EtOH} 226–233, 256, 358 mμ (ε 24,000, 53,000, 14,000).

Anal. Calcd for $C_{13}H_{10}N_2O$: C, 74.27; H, 4.79; N, 13.33. Found: C, 74.17; H, 4.73; N, 13.53.

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1-Substitution in 2-Methyl-4(5)-nitroimidazole. I. Synthesis of Compounds with Potential Antitrichomonal Activity

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In view of the known antitrichomonal activity of some 1-substituted 2-methyl-4(5)-nitroimidazoles, a series of 4-nitro and 5-nitro isomers was synthesized and evaluated for their activity against *Trichomonas vaginalis*. Earlier authors[2-4] have prepared some other series of such compounds and determined[3,5] some structure–activity relationships.

The methods employed and characteristic data for the compounds synthesized are shown in Tables I and II. The starting material for all preparations was 2-methyl-4(5)-nitroimidazole (III).[6] A number of 1-substituted 2-methyl-5-nitroimidazoles was obtained by a general method which consisted of the use of one of the lower carboxylic acids with high polarity, which activates nucleophilic agents and allows the formation of 5-nitro isomers only.[7] The influence of the carboxylic acids is not yet clearly explained,[8] but a greater ratio of these reagents, empirically established, is always necessary to give rise to only 5-nitro isomers (see procedure A). 4-Nitro isomers were obtained when a molar excess of alkylating agents was used without addition of carboxylic acid, or when the solution of the sodium salt of III was employed according to an earlier described procedure.[9] Another synthetic peculiarity is found in procedure E where a supersaturated solution of potassium iodide in methyl isobutyl ketone is found to give significantly better results than the classical Finkelstein method.[10]

In preparing picrates of the compounds listed in Tables I and II, we were able to confirm the observation[11] that only 5-nitro isomers form stable hydrochlorides and picrates (see Table I). This fact could serve to distinguish 5-nitro and 4-nitro isomers if carboxyl functions are absent. Another possibility for such distinction is offered by the nmr spectra of these isomers if there is at least one proton on the α-carbon of the substituting side chain attached to ring nitrogen.

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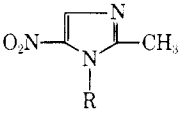
TABLE I

1-SUBSTITUTED 2-METHYL-5-NITROIMIDAZOLES, THEIR ANTITRICHOMONAL ACTIVITY AND TOXICITY

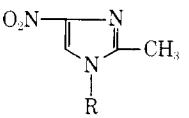


No.	R	Method	Yield,[d] %	Recrystn solvent	Mp, °C	Picrates mp, °C	Formula[g]	Min lethal concn, g/ml × 10^{-3} [f]	LD_{50}, mg/kg × 10^{-2}
1	CH_2CH_2Cl[a]	A[c]	15	H_2O–EtOH (2:1)	78.5–79.5	139–140	$C_6H_5ClN_3O_2$	1:200	10
2	CH_2CH_2Br	A,[c] C	25.5, 65	H_2O–EtOH (2:1)	80–81	145–146	$C_6H_5BrN_3O_2$	1:200	7.3
3	CH_2CH_2I	D	90	H_2O–EtOH (2:1)	103–104	148.5–150	$C_6H_5IN_3O_2$	1:45	3.2
4	$CH_2COOC_2H_5$[b]	A[c]	12	H_2O	72–73	129–130	$C_8H_{11}N_3O_4$	1:400	38
5	CH_2COOH[b]	G	92	H_2O	179–180	...[e]	$C_6H_7N_3O_4$	1:60	6.5
6	CH_2CN[b]	A[c]	14	H_2O	92–93	133–134	$C_6H_6N_4O_2$	1:700	5.0
7	$CH_2CH_2OC_6H_5$	A[c]	39	EtOH	105–106	182–183	$C_{12}H_{13}N_3O_3$	1:250	7.5
8	$CH_2CH_2OCH_2CH_3$	A[c]	72	H_2O	62.5–63.5	134–135	$C_8H_{13}N_3O_3$[h]	1:350	15
9	$CH_2CH_2OCH_2CH_2Cl$	A[c]	55	H_2O–EtOH (1:2)	79–80	114–115	$C_8H_{12}ClN_3O_3$	1:300	6.3
10	$CH_2CH_2OCH_2CH_2I$	D	85	H_2O–MeOH (1:3)	79–81	116–117	$C_8H_{12}IN_3O_3$	1:500	3.5
11	$CH_2CH_2OCH_2CH_2OH$	F	68	MeOH–petr ether (60–80°)	62–63	107–108	$C_8H_{13}N_3O_4$	1:600	9.5

[a] Prepared first by C. Cosar, *et al.*,[2] by a different method. [b] First described by May Co. Baker Ltd., Belgian Patent 639,372 (1964); *Chem. Abstr.*, **62**, 9144*h* (1965), but was obtained *via* CrO_3 oxidation of I. [c] Alkylating agents and carboxylic acids employed: **1**, 43.0 g (25.5 ml, 0.30 mole) of 1-bromo-2-chloroethane, 29.6 g (30 ml) of propionic acid, and 30.0 g (25 ml) of nitrobenzene; **2**, 84.5 g (38.8 ml, 0.45 mole) of 1,2-dibromoethane, 21 g (20.0 ml) of AcOH; **4**, 49.0 g (42.4 ml, 0.40 mole) of ethyl α-chloroacetate, 19.8 g (20.0 ml) of propionic acid; **6**, 90.2 g (25.4 ml, 0.40 mole) of chloroacetonitrile, 19.8 g (20.0 ml) of propionic acid; **7**, 40.0 g (0.18 mole) of β-bromophenetole, 21 g (20.0 ml) of AcOH; **8**, 45.8 g (33.8 ml, 0.3 mole) of β-bromoethyl ethyl ether, 42 ml (43.5 g) of AcOH; **9**, 58.5 g (48 ml, 0.41 mole) of β,β′-dichlorodiethyl ether, 11 g (9 ml) of formic acid. [d] Where several values are indicated, these correspond to the methods mentioned. [e] Picrate cannot be obtained because of the presence of carboxyl group. [f] Minimum lethal concentration for I, 1:1000 (g/ml × 10^{-3}); LD_{50} = 42 (mg/kg × 10^{-2}). [g] Analytical results obtained for C, H, and N were within ±0.4% of the theoretical values unless listed otherwise. [h] *Anal.* C, H; N: calcd, 21.10; found, 20.67.

TABLE II

1-SUBSTITUTED 2-METHYL-4-NITROIMIDAZOLES, THEIR ANTITRICHOMONAL ACTIVITY AND TOXICITY



No.	R	Method	Yield,[d] %	Recrystn solvent	Mp, °C	Formula[g]	Min lethal concn, g/ml × 10^{-3} [f]	LD_{50}, mg/kg × 10^{-2}
12	CH_2CH_2Cl	E[a]	65	H_2O–EtOH (2:1)	97–98	$C_6H_5ClN_3O_2$	1:1.5	8.5
13	CH_2CH_2Br	C, E[a]	65, 70	H_2O–EtOH (2:1)	100–101	$C_6H_5BrN_3O_2$	1:1	3.5
14	CH_2CH_2I	D	90	H_2O–EtOH (2:1)	121–122	$C_6H_5IN_3O_2$[h]	1:1.5	2.8
15	$CH_2COOC_2H_5$[b]	B, from **16**[c]	63,[b] 92	H_2O	111–112	$C_8H_{11}N_3O_4$	1:1	65
16	CH_2COOH	B, G	25, 92	H_2O	247–248[e]	$C_6H_7N_3O_4$	1:1	25
17	CH_2CN	B, E[a]	28, 38	H_2O	125–126	$C_6H_6N_4O_2$	0	8.0
18	$CH_2CH_2OC_6H_5$	E[a]	60	EtOH	136–137	$C_{12}H_{13}N_3O_3$	1:1.2	30
19	$CH_2CH_2OCH_2CH_3$	B, E[a]	14, 70	H_2O	100–101	$C_8H_{13}N_3O_3$[i]	1:2	18
20	$CH_2CH_2OCH_2CH_2Cl$	B, E[a]	40, 65	H_2O–EtOH (1:2)	101–102	$C_8H_{12}ClN_3O_3$	1:1	7.5
21	$CH_2CH_2OCH_2CH_2I$	D	85	H_2O–EtOH (1:3)	67–68	$C_8H_{12}IN_3O_3$	1:1	3.5
22	$CH_2CH_2OCH_2CH_2OH$	F	68	H_2O	93–94	$C_8H_{13}N_3O_4$	1:1.6	23

[a] Alkylating agents: **12**, 1-bromo-2-chloroethane; **13**, 1,2-dibromoethane; **17**, α-chloroacetonitrile; **18**, β-bromophenetole; **19**, β-bromoethyl ethyl ether; **20**, β,β′-dichlorodiethyl ether. [b] Prepared by method B by Cosar, *et al.*[2] [c] Esterification of **16** in HCl–ethanol. [d] Where several values are indicated, these correspond to the methods mentioned. [e] Slow decomposition above 240°. [f] Minimum lethal concentration for II, 1:1 (g/ml × 10^{-3}); LD_{50} = 58 mg/kg × 10^{-2}. [g] C, H, N analyses: see Table I, footnote *g*. [h] *Anal.* C: calcd, 25.63; found, 26.13. [i] *Anal.* H: calcd, 6.58; found, 6.01.

δ values of 5-nitro isomers are about 0.45 ppm greater than those of the corresponding 4-nitro isomers,[12] because of the greater deshielding effect of the 5-nitro group as compared with that in position 4. The polarographic measurements served as the third analytical method for the differentiation of the isomeric compounds. It was found that half-wave potentials were dependent only upon the position of the nitro group.[13] Ir spectra of the isomeric compounds showed a characteristic difference for 4(5)-CH out-of-plane bending vibration. The difference between characteristic frequencies was about 11 cm^{-1}. The frequency of the C–H band of the 4-nitro isomers lies at 754–755 cm^{-1}, and of the 5-nitro isomers at 743–744 cm^{-1}.

Pharmacology. Methods.—Acute toxicities were determined in mice weighing 15–20 g. The viscous suspension containing 1 g of the substance in 5 ml of water was administered orally. All deaths occurring during the following 24 hr after administration of the drug were recorded for the estimation of LD_{50} values. *In vitro* trichomonacidal activity was determined by test-

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ing the inhibitory effect of the different concentrations of the compound against *Trichomonas vaginalis* in vaginal secretion. Compounds I and II served as the reference substances. I is 1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole, a well-known antitrichomonal agent,[2,14] II is its 4-nitro isomer, the relative activity of which has been established.[5,6] Compounds I, II, and **1–22** were dissolved in saline in different concentrations and the minimum concentration which suppressed completely the growth of trichomonads at 24 and 37° was determined. The end points were ascertained by microscopic examination of the culture, death being indicated by the characteristic morphological change of the cells and complete cessation of motility. The assays were carried out in Löefler's nutrient medium at 24 and 37°.

Results (see Tables I and II).—Preliminary investigations confirmed the observation that the 5-nitro isomers regularly had higher activity than the corresponding 4-nitro isomers.[2] However, the difference in the activities of the isomeric compounds was found to be far greater in favor of 5-nitro isomers than noticed earlier.[3a,15,16] In addition, the exchange of the functional group in the side chain on N^1 led to a noteworthy alteration of the activity. This effect was particularly noticeable for the 5-nitro isomers. The compounds containing nitrile, ester, or ether groups (**4**, **6**, **7**, **9–11**) showed distinctly increased activity as compared with I.

Experimental Section[17]

Syntheses of the compounds in Tables I and II were carried out by procedures A–G, which are illustrated below.

Procedure A.—Compound III (6.3 g, 0.05 mole), 0.45 mole of alkylating agent, and the particular carboxylic acid (see Table I, footnote *c*) were heated at reflux temperature for 12–18 hr. Minimum reflux temperature should be about 120–125°; therefore, in the preparation of **1** nitrobenzene was added to the reaction mixture. After removing the liquid components *in vacuo*, the residue was dissolved in hot H_2O, filtered (charcoal), and cooled to precipitate unchanged III. The filtrate was basified, and the crude product which separated was collected and dried. Further recrystallizations were carried out in the solvents listed in Table I.

Procedure B.—A mixture of 6.3 g (0.05 mole) of III and 0.3–0.5 mole of alkylating agent (Table II, footnote *b*) was heated at reflux temperature for 12–20 hr. Excess reagent was evaporated *in vacuo* and the residue was recrystallized from hot H_2O (charcoal) giving the crude product. Specific separation of **16** from unreacted III consisted in dissolving the mixture obtained in a fivefold amount of cold H_2O and changing the pH from 3 to 7.3 to dissolve **16** in the form of its Na salt. Undissolved III was filtered, and the filtrate was acidified again to give crude **16**.

Procedure C.—Compound **9** (10.0 g, 0.0428 mole) and 145 g (100 ml, 1.78 moles) of 48% HBr were heated at reflux temperature for 14 hr. 1-Bromo-2-chloroethane formed by cleavage of **9** was continuously separated by drawing it off the bottom of the flask. The crude by-product was washed (H_2O), dried (Na_2SO_4), and redistilled to give 2.2 ml (3.72 g) of material, $n^{20}D$ 1.5250–1.5300. After the reaction was finished HBr was evaporated *in vacuo* and the residue recrystallized from 100 ml of hot H_2O (charcoal). The filtrate was basified to pH 10 and the precipitate was collected to furnish the crude product. Compound **13** was prepared by the same procedure from **20**.

Procedure D.—The substance to be iodinated and KI were slurried in a molar ratio of 2:1 with about the tenfold amount of methyl isobutyl ketone. The reaction mixture was stirred vigorously to prevent sedimentation of the inorganic salt, and heated at reflux temperature. To prepare **3** and **14** heating was carried out for 8 hr, and to prepare **10** and **21**, for 18 hr. When the reaction was finished, the solvent was evaporated *in vacuo*, inorganic salts and product were filtered and washed with cold H_2O, and the remaining crude product was recrystallized (see Tables I and II).

Procedure E.—Sodium ethoxide (2.77 g, 0.05 mole) in 50 ml of absolute EtOH and 6.3 g (0.05 mole) of III were heated to complete solution. The solution of the sodium salt of III thus obtained was cooled and 0.05 mole of the particular alkylating agent was added (see Table II, footnote *a*). The reaction mixture was refluxed for 24 hr and then evaporated to dryness. The residue was treated in one of the following ways. (a) To isolate **12**, **13**, and **20** the residue was slurried with $CHCl_3$, undissolved III and inorganic salts were altered, and the filtrate was concentrated to dryness. The crude products thus obtained were recrystallized according to the Table II. (b) Compound **17** was extracted from the residue with MeCN. (c) Compounds **18** and **19** were obtained recrystallizing the residue from H_2O.

Procedure F.—A solution of 0.05 mole of a substance which was to be hydrolyzed (**9** or **20**) in 35 ml (39.5 g) of formamide and 4.75 ml (5 g) of 46% formic acid was heated at 140–150° for 4 hr. When the reaction was finished formamide was evaporated at 80–90° (0.5 mm) and the residue recrystallized.

Procedure G.—Compounds **4** or **15** (4.2 g, 0.02 mole) were dissolved in 50 ml of 5% NaOH, shaken, and heated on a steam bath until a slightly red color appeared. Heating was discontinued, the solution was cooled immediately and acidified with concentrated HCl to pH 1, and the product was filtered and dried.

(14) Flagyl®, Clont®, Efloran®.

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(17) All melting points were determined using a Bötius Mikroheiztisch apparatus and are corrected. Elemental microanalyses were carried out by microanalytical laboratory, Department of Organic Chemistry, Faculty of Pharmacy and Biology, University of Zagreb. Where analyses are indicated only by symbols of the elements, analytical results obtained for those elements were within ±0.4% of the theoretical values.

Resolution and Racemization of *dl*-Tetramisole, *dl*-6-Phenyl-2,3,5,6-tetrahydroimidazo-[2,1-*b*]thiazole

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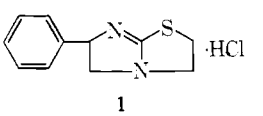
dl-6-Phenyl-2,3,5,6-tetrahydroimidazo[2,1-*b*]thiazole hydrochloride (tetramisole) (**1**) has been found to be a broad-spectrum anthelmintic compound extremely effective for the treatment of helminthiases in domestic animals. Methods for the preparation of this *dl* compound and reports of useful biological activity have been published.[1]



1

(1) (a) A. H. M. Raeymaekers, F. T. N. Allewijn, J. Vandenberk, P. J. A. Demoen, T. T. T. V. Ottenwert, and P. A. J. Janssen, *J. Med. Chem.*, **9**, 545 (1966); (b) D. C. I. Thienpont, O. F. J. Vanporijs, A. H. M. Raeymaekers, J. Vandenberk, P. J. A. Demoen, F. T. N. Allewijn, R. P. H. Marsboom, C. J. E. Niemegueers, K. H. L. Schellekens, and P. A. J. Janssen, *Nature*, **209**, 1084 (1966); (c) J. W. Pankhurst and D. O. Sutton, *Vet. Record*, **79**, 166 (1966); (d) J. S. Remders, *Neth. J. Vet. Sci.*, **91**, 967 (1966); (e) J. K. Walley, *Vet. Record*, **78**, 406 (1966); (f) L. D. Spicer, M. W. Bullock, M. Garber, W. Groth, J. J. Hand, D. W. Long, J. L. Sawyer, and R. S. Wayne, submitted for publication.

The *dl* compound was resolved with *d*-10-camphorsulfonic acid when chloroform was used as the solvent. The *d*-(+)-6-phenyl-1,2,3,5,6-tetrahydroimidazothiazole *d*-10-camphorsulfonate crystallized as a trisolvate in 90% yield. When the mother liquor containing the soluble *l*-amine salt was concentrated and treated with hot acetone, a racemic salt crystallized which contained the balance of the *d*-amine salt and left in solution essentially pure *l*-amine salt. The *l*-amine salt crystallized on cooling.

A novel procedure for effecting the resolution took advantage of the fact that in solvents other than chloroform the *dl* base crystallized with *d*-10-camphorsulfonic acid as a racemic compound which is less soluble than the resolved salts. When the *dl* base was added to a chloroform solution of either the *d*- or *l*-amine *d*-10-camphorsulfonates, *which need not be optically pure*, and toluene was added, racemic amine *d*-10-camphorsulfonate crystallizes leaving only optically pure amine in solution. The optimum amount of racemic amine to add is slightly less than equimolar to compensate for the cocrystallization of a small amount of the resolved salt with the racemic salt. This method yielded both the *d* and the *l* bases in high yield and in high optical purity, and simultaneously recovered the resolving acid as the racemic amine salt which can be recycled.

This general method can be employed to precipitate the *dl* component as the camphorsulfonate from an optically impure amine and leave only optically pure amine in solution from which it is easily precipitated as the hydrochloride.

dl-10-Camphorsulfonic acid can be resolved by the optically active amines. The general method of Corrodi and Hardegger[2] is applicable although not as convenient as using both forms of the amine which are now readily available.

We have found that the *d* and *l* forms of tetramisole are approximately equal in acute toxicity and that the activity toward those nematodes tested predominated in the levo isomer. Tables I and II illustrate these toxicity and activity relationships.

Raeymaekers and collaborators[3] have synthesized the isomers from optically active phenylethylenediamine and established that the levo form is sinistral. Racemization of the uninteresting dextrorotatory isomer would be important in converting it to a more anthelmintically active form. The asymmetric center of **1** is the benzylic carbon in position 6. The 6-hydrogen would appear to be the most acidic hydrogen in the molecule and formation of an anion at this position would be predicted to racemize the compound. Since tetramisole is hydrolyzed in basic solutions[1] the use of strong bases in solvents that have no ionizable hydrogen would be essential. The free base was found to be racemized when heated neat at 100° in the presence of potassium *t*-butoxide. The amine is racemized immediately when a solution in dimethyl sulfoxide is treated with the sodium salt of dimethyl sulfoxide. Racemizations with sodium methoxide in dimethyl sulfoxide, potassium *t*-butoxide in dimethylformamide, and *n*-butyllithium in benzene were less satisfactory as more by-products were formed.

TABLE I

BIOLOGICAL ACTIVITY OF *l*-(−), *d*-(+), AND *dl*-TETRAMISOLE AGAINST *Nematospiroides dubius* IN MICE

Oral dose, mg/kg	% efficacy		
	levo	dextro–levo	dextro
2	0	..	..
4	47	..	..
6	69	10	..
8	79	31	..
12	97	61	..
16	..	83	0
32	..	..	22
48	..	..	36

TABLE II

ACUTE ORAL TOXICITY OF *l*-(−), *d*-(+), AND *dl*-TETRAMISOLE IN MICE

Dose, mg/kg	Dead/total mice		
	levo	dextro–levo	dextro
100	0/10	1/10	1/10
150	16/30	8/30	8/10
200	16/20	15/20	8/10
300	9/10	10/10	9/10

(2) H. Corrodi and E. Hardegger, *Helv. Chim. Acta*, **40**, 193 (1957).

Experimental Section

Melting points are corrected. The optical rotations were measured with a manually operated polarimeter in 2-dm tubes. Evaporations, unless otherwise specified, were done with a rotary evaporator.

DL-6-Phenyl-2,3,5,6-tetrahydroimidazo[2,1-*b*]thiazole *d*-10-Camphorsulfonate.—A suspension of 227 g (0.938 mole) of *dl*-6-phenyl-2,3,5,6-tetrahydroimidazo[2,1-*b*]thiazole hydrochloride in 400 ml of water and 400 ml of CH_2Cl_2 was treated with a solution of 45 g (1.09 moles) of 97% NaOH in 200 ml of water and ice. The organic layer was separated and dried (K_2CO_3), filtered, and treated with 217.9 g (0.938 mole) of *d*-10-camphorsulfonic acid in portions which dissolved rapidly with the liberation of heat. Toluene (800 ml) was added, the CH_2Cl_2 was distilled, and the salt was recovered by filtration and washed with toluene and with hexane. The yield was 408 g (0.935 mole, 99%), mp 195–197°, $[\alpha]^{25}D$ +14.0° (*c* 11, H_2O).

Anal. Calcd for $C_{21}H_{28}N_2O_5S_2$: N, 6.42; S, 14.66. Found: N, 6.13; S, 14.36.

***d*-(+)-6-Phenyl-2,3,5,6-tetrahydroimidazo[2,1-*b*]thiazole *d*-10-Camphorsulfonate.**—A warm solution of 204.3 g (1 mole) of *dl*-**1** (**1a**) and 232.3 g (1 mole) of *d*-10-camphorsulfonic acid in 1750 ml of $CHCl_3$ was allowed to crystallize overnight at −28°. The $CHCl_3$ solvate was recovered by filtration and washed with 400 ml of ice-cold $CHCl_3$. The solvate (somewhat hygroscopic) was dried several hours in dry N_2 and then in air overnight. The yield of nonsolvated material was 202.5 g (92.8%), mp 139–140°, $[\alpha]^{25}D$ +82.6° (*c* 16, H_2O). Material recrystallized from $CHCl_3$ had mp 140–141° and $[\alpha]^{25}D$ +83.0° (*c* 15, H_2O).

Anal. Calcd for $C_{21}H_{28}N_2O_5S_2$: N, 6.42; S, 14.66. Found: N, 6.23; S, 14.28.

The $CHCl_3$ solvate is unstable in air. The composition was estimated to be that of a trisolvate by nmr spectroscopy on freshly prepared samples.

***l*-(−)-6-Phenyl-2,3,5,6-tetrahydroimidazo[2,1-*b*]thiazole *d*-10-Camphorsulfonate.**—The mother liquor from the above preparation (containing *ca.* 10% of *d*) was evaporated to a syrup which was approximately one-half $CHCl_3$ by weight and treated with 1500 ml of hot Me_2CO. When maintained near the boiling point for about 10 min a solid crystallized. The crystals (*dl*-salt enriched with *l*-salt) were collected by filtration of the hot acetone mixture and washed with 200 ml of hot acetone. The optically impure salt weighed 24.2 g (0.055 mole), mp 186–192°, $[\alpha]^{25}D$ −14.7° (*c* 16, H_2O). It can be resolved by recrystallization ($CHCl_3$). Refrigeration of the acetone filtrate at −15° overnight

(3) A. H. M. Raeymaekers, L. F. C. Roevens, and R. A. J. Janssen, *Tetrahedron Letters*, 1467 (1967).

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COMMUNICATION

The Syntheses and Crystal Structures of Metronidazole-derived Compounds

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Abstract Metronidazole (MET-OH), widely used as an antibacterial agent, is found to have some side effects on human bodies. Due to these disadvantages, people have been looking for its modification compounds for substituents. In this article, four MET-OH derivatives were designed, prepared, and structurally characterized by single crystal X-ray diffraction. These compounds are MET-OTs (**1**), MET-Br (**2**), MET-Cl (**3**), and MET-I (**4**). X-ray structure analyses revealed that, **1** crystallized in the monoclinic system with space group $P2_1/c$, with $a = 16.1178$, $b = 7.5473$, $c = 13.4161$ Å, $V = 1520.3$ Å^3, $\beta = 111.3210^o$ and $Z = 4$. **2** crystallized in the monoclinic system with space group $P2_1/c$, with $a = 12.079$, $b = 11.089$, $c = 6.380$ Å, $V = 847.1$ Å^3, $\beta = 97.57^o$ and $Z = 4$. **3** crystallized in the monoclinic system with space group $P2_1/c$, with $a = 12.098$, $b = 11.007$, $c = 6.295$ Å, $V = 830.3$ Å^3, $\beta = 97.886^o$ and $Z = 4$. **4** crystallized in the triclinic system with space group $P1$, with $a = 6.192$, $b = 7.740$, $c = 10.001$ Å, $V = 457.9$ Å^3, $\alpha = 89.073$, $\beta = 86.903$, $\gamma = 73.097^o$ and $Z = 2$.

Keywords Metronidazole (MET-OH) · Crystal structure · Halogenations · 4-Methyl-benzenesufonyl chloride

Introduction

MET-OH (Scheme 1) is one of the nitroimidazole derivatives that are an extremely important class of compounds. It is extensively used in the treatment of anaerobic infections and is under continuing investigation [1, 2]. Though widely used as an antibacterial medicine, it also has some side effects [3]. For example, the metabolism products of MET-OH can combine with nerve cell RNH, which may strengthen the excitement of the bronchus myocardial nerve fiber controlled by the RNH, extend the capillary vessel and increase the transparence, consequently it would cause the nervous system, respiration system, cardiovascular system, immune system(... etc. to work abnormally.

In order to overcome its disadvantages, a variety of compounds related to MET-OH with less toxicity needed to be synthesized. There is a hydroxyl group in the molecule of MET-OH, thus there are definitely lots of modifying measurements starting from this group. For example, changing the structure to the Tinidazole (Scheme 2) can make it more effective in the treatment of anaerobic fungus and diseases infected by ectosarc [4, 5]. However, as it is not so easy to change the hydroxyl directly into other functional groups, the hydroxyl group can be designed to be remodeled to -OTs group or halogen group firstly, consequently, the introduction of other more complex groups would become convenient. There some reports on the halogenations of the hydroxyl group in the literature [6–8], but the reaction condition is rather strict and the yield is rather low, here a more convenient and effective way to replace the hydroxyl group is presented and the structures of four intermediates (Scheme 3) were determined by X-ray crystallography report.

Experimental

Materials and Physical Measurements

All chemicals and reagents used in current study were of analytical grade. MET-OH was purchased from Chan Zhou

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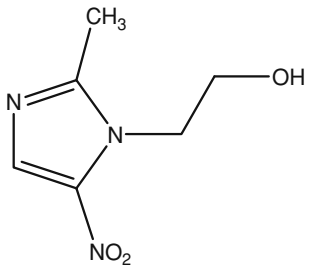
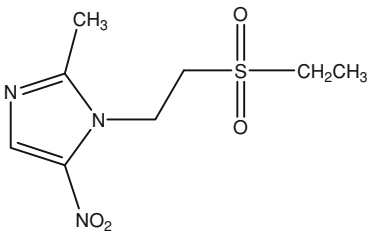
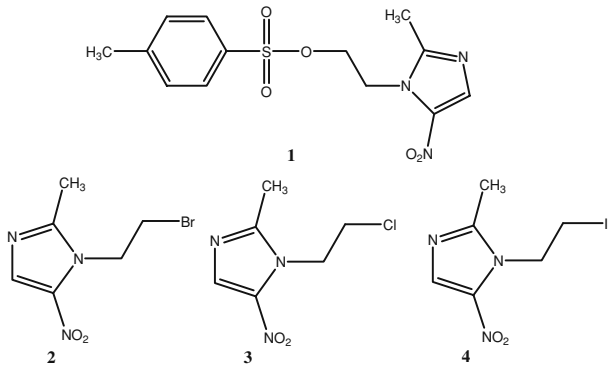


Scheme 1 Structure of Metronidazole



Scheme 2 Structure of Tinidazole



Scheme 3 Structures of compounds **1**–**4**

Dongsheng Company, Chan Zhou, P. R. China. TLC was run on the silica gel coated aluminum sheets (silica gel 60 GF_{254}, E. Merk, Germany) and visualized in UV light (254 nm). All the NMR spectra were recorded on a Bruker DRX 500 model Spectrometer in either DMSO-d_6 or $CDCl_3$. Chemical shifts (δ) for ^{1}H-NMR spectra are reported in parts per million to residual solvent protons. Melting points were measured on a Boetius micro melting point apparatus.

Preparations

Synthesis of 2-(2-methyl-5-nitro-imidazol-1-yl)-ethyl ester toluene-4-sulfonate (1) (Scheme 4)

MET-OH (3.14 g, 20 mmol) and Et_3N (3.0 mL, 22 mmol) were dissolved in CH_2Cl_2 (20 mL), and 4-methyl-benzenesufonyl chloride (3.83 g, 20.1 mmol) in CH_2Cl_2 (10 mL) was added. The reaction mixture was stirred at 0 °C for 5 h, and 30 mL of ice water was added, the layer was separated, and the aqueous layer was extracted with ethyl acetate (2 × 30 mL). The organic layer was combined and washed with saturated $NaHCO_3$, and dried with anhydrous Na_2SO_4 for 0.5 h. Removal of the solvent gave a slight-yellow crystal of compound **1** (5.89 g, 18.1 mmol). Yield: 90%; Mp: 155–157 °C; ^{1}H NMR (DMSO-d_6): 2.40 (s, 3H, Ar–CH_3); 2.49 (s, 3H, –CH_3–); 4.38 (t, J = 9.72 Hz, 2H, N–CH_2–); 4.55 (t, J = 9.80 Hz, 2H, O–CH_2–); 7.38 (d, J = 7.97 Hz, 2H), 7.59 (d, J = 8.30 Hz, 2H); 7.92 (s, 1H).

Synthesis of 1-(2-bromoethyl)-2-methyl-5-nitro-1H-imidazole (2) (Scheme 4)

Compound **1** (6.54 g, 20.1 mmol) was dissolved in 30 mL of anhydrous DMF and the solution was stirred at 100 °C for 15 min. Then NaBr (5.2 g, 50 mmol) in anhydrous DMF (20 mL) was carefully added, after the mixed solution was stirred at 100 °C for 4 h, the mixture was cooled to 20 °C, and the solvent was removed under reduced pressure. The residue was suspended in EtOH (100 mL) and filtered, the solvent was removed under reduced pressure, and the residue was chromatographed. Elution with EtOAc gave an oil which recrystallized from EtOAc/petroleum ether (3/1) to give 1-(2-bromoethyl)-2-methyl-5-nitro-1H-imidazole (**2**) (3.28 g, 14 mmol). Mp: 78.5–79.5 °C; Yield: 70%; ^{1}H NMR (DMSO-d_6): 2.50 (s, 3H, –CH_3–); 3.51 (t, J = 8.68 Hz, 2H, N–CH_2–); 4.61 (t, J = 8.51 Hz, 2H, –CH_2–); 8.05 (s, 1H).

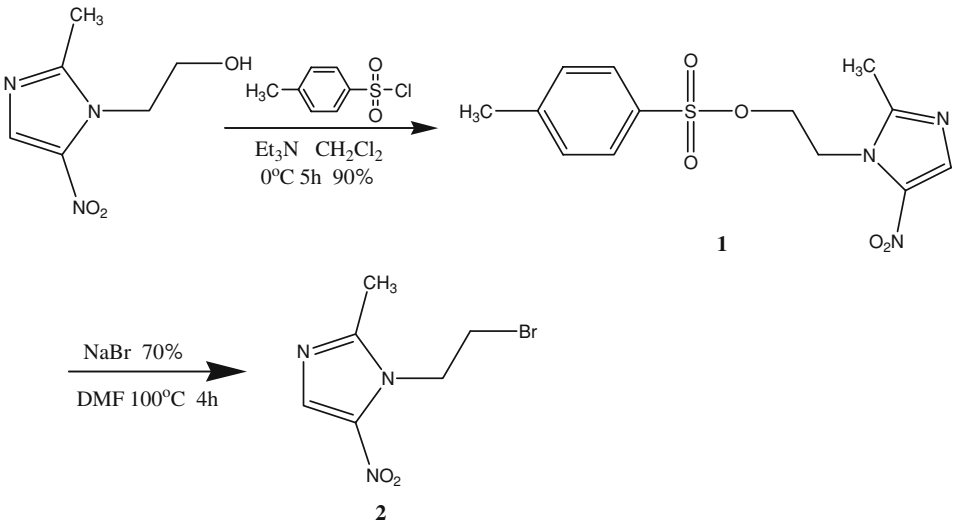
Synthesis of 1- (2-chloroethyl)-2-methyl-5-nitro-1H-imidazole (3) (Scheme 5)

MET-Cl was synthesized as the literary method [9] with slight changes. MET-OH (4.71 g, 30 mmol) was dissolved in $SOCl_2$ (11 mL, 0.15 mol), and the solution was stirred at 70 °C for 6 h and cooled. The solvents and excess $SOCl_2$ were then removed under reduced pressure. Compound **3** (4.98 g, 26.3 mmol) was obtained as a yellow crystal. Yield: 88%; Mp: 78.5–79.5 °C; ^{1}H NMR (DMSO-d_6): 2.49 (s, 3H, –CH_3–); 3.69 (t, J = 9.75 Hz, 2H, N–CH_2–); 4.95 (t, J = 9.88 Hz, 2H, –CH_2–); 8.35 (s, 1H).

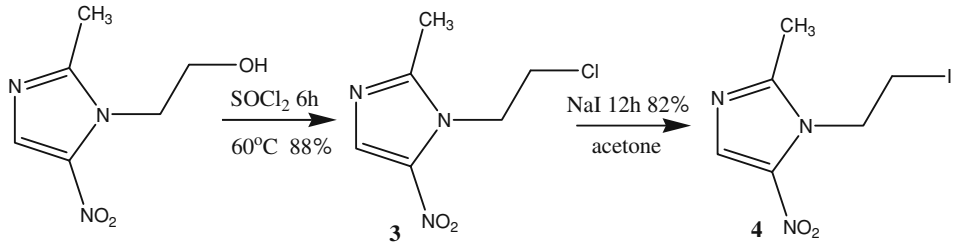
Synthesis of 1-(2-iodo-ethyl)-2-methyl-5-nitro-1H-imidazole (4) (Scheme 5)

MET-I was synthesized as the literary method [10] with slight changes. Compound **3** (10.43 g, 55.0 mmol) was dissolved in 30 mL of anhydrous acetone; the solution was stirred at 90 °C for 15 min. Then NaI (9.0 g, 60.0 mmol) in anhydrous acetone (20 mL) was carefully

Scheme 4 The synthetic route of compounds **1** and **2**



Scheme 5 The synthetic route of compounds **3** and **4**



added. After the mixed solution was stirred at 90 °C for 12 h, the solvents were removed and the residue recrystallized from chloroform gave compound **4** (12.70 g, 45.2 mmol) as a yellow solid. Yield: 82%; Mp: 78.5–79.5 °C; ^{1}H NMR (DMSO-d_6): 2.59 (s, 3H, –CH_3–); 3.89 (t, J = 11.25 Hz, 2H, N–CH_2–); 4.64 (t, J = 11.15 Hz, 2H, –CH_2–); 7.99 (s, 1H).

Results and Discussion

Syntheses of the Four MET Derivatives

In this paper, four MET derivatives: **1**, **2**, **3**, and **4** were synthesized from the metronidazole. Treatment of MET-OH with TsCl gave **1**, treatment of **1** with sodium bromide in DMF gave **2**, treatment of MET with thionychloride gave **3**, treatment of **3** with sodium iododide in anhydrous acetone gave **4**.

When the hydroxyl group was changed to -OTs, the ratio of the starting materials had great effect on the yield of the reaction. Both triethylamine and pyridine could act as the base in the reaction, the less poisonous triethylamine is chosen as the base in our study. The use of equivalent of triethylamine in the reaction is the most optimal, and dichloromethane is found to be the best solvent for the reaction that produced the highest yield. The bromization reaction with the intermediate **1** has also been carried out, the cheap sodium bromide is used as the brominating agent in DMF in place of phosphorus tribromide in the organic solvent, in thus condition the reaction could be easier to handle, which also supplied a new valuable method for the bromization.

As to the chlorination of the hydroxyl group, dichlorosulfoxide has been used as the chlorination reagent, the reaction is homogeneous and the reaction condition is milder in comparison to the literatures, the selectivity is also very good. The less poisonous dichlorosulfoxide compared to benzene is employed as the solvent and thus the reaction proceed under a comparatively low temperature, which made the reaction more controllable and milder.

Crystal Structures of Compounds **1–4**

Diffraction intensities for complexes **1–4** were collected on a CCD area detector diffractmeter equipped with graphite-monochromated Mo Kα ($\lambda = 0.71073$ Å) radiation. The intensities were collected using the 2θ scan mode with variable scan speed. Crystal data were corrected for Lorentz and polarization effects during data reduction using XSCANS [11–13]. All the non-hydrogen atoms were refined anisotropically. Hydrogen atoms were generated geometrically and allowed to ride on their parent carbon atoms. Analytical expressions of neutral-atom scattering

Table 1 Crystallographic and experimental data for compounds **1–4**

Compound	**1**	**2**	**3**	**4**
Formula	C_{13} H_{15} N_3 O_5 S	$C_6H_8BrN_3O_2$	$C_6H_8ClN_3O_2$	$C_6H_8IN_3O_2$
FW	325.34	234.06	189.60	281.05
Crystal shape/color	Block/colorless	Prism/colorless	Prism/colorless	Prism/Pale yellow
Crystal size/mm	0.20 × 0.10 × 0.10	0.40 × 0.30 × 0.10	0.38 × 0.35 × 0.30	0.40 × 0.35 × 0.35
Crystal system	Monoclinic	Monoclinic	Monoclinic	Triclinic
Space group	*P2₁/c*	*P2₁/c*	*P2₁/c*	P1
a/Å	16.1178	12.079	12.098	6.192
b/Å	7.5473	11.089	11.007	7.740
c/Å	13.4161	6.380	6.295	10.001
α/°				89.073
β/°	111.3210(10)	97.57	97.886	86.903
γ /°				73.097
V /Å^3	1520.3	847.1	830.3	457.9
Z	4	4	4	2
T/ K	292	293	298	298
μ/mm^{-1}(Mo-K*α*)	0.240	4.815	0.42	3.46
D_x/mg m^{-3}	1.421	1.835	1.517	2.039
Reflections/parameters	3469/201	1647/109	1895/110	1553/110
F(000)	680	464	429	675
T_{max}	0.9764	0.6445	0.890	0.298
T_{min}	0.9536	0.2490	0.850	0.265
θ range /°	2.71/27.50	1.70/25.95	1.70/28.33	2.04/25.00
Index range (*h*, *k*, *l*)	−20/20, −9/9, −17/17	−14/14, −13/0, 0/7	−16/14, −14/14, −6/8	−7/7, −7/9, −10/11
Reflections collected	3469	1647	1895	1553
Independent reflections	1647	960	1328	1410
Goodness of fit on F^2	1.051	1.050	1.004	1.060
R_1, wR_2 [I ≥ 2*σ*(I)][a)]	0.0485, 0.0598	0.0659, 0.1251	0.0503, 0.0711	0.0391, 0.0423

factors were employed, and anomalous dispersion corrections were incorporated. The crystallographic data were listed in Table 1.

Figures 1–4 gives a perspective view of the molecular structures of **1–4** together with the atomic labeling system. Counpound **1–3** crystallized monoclinically with space group *P2₁/c*, while MET-I **4** crystallized in the triclinic system with space group *P1*. For compound **1**, the imidazole ring (plane I) and the benzene ring (plane II) both are well-defined planes with an average deviation of 0.0010 Å for the former and 0.0051 Å for the later. The dihedral angles between the basal planes are as follows: 3.9° between plane I and the nitro plane; 10.3° between plane I and plane II; and 7.3° between the nitro plane and plane II.

In the molecules of compound **2–4**, the imidazole ring in each molecule is planar, with an average deviation of 0.0023 Å for compound **2**, 0.0016 Å for compound **3** and 0.0079 Å for compound **4**. The nitro N atom lays 0.059 Å of **2** (0.060 Å for **3**, 0.017 Å for **4**) above the plane. The two other groups attached to the ring are located on the opposite side of the plane, with displacements of 0.097 (for

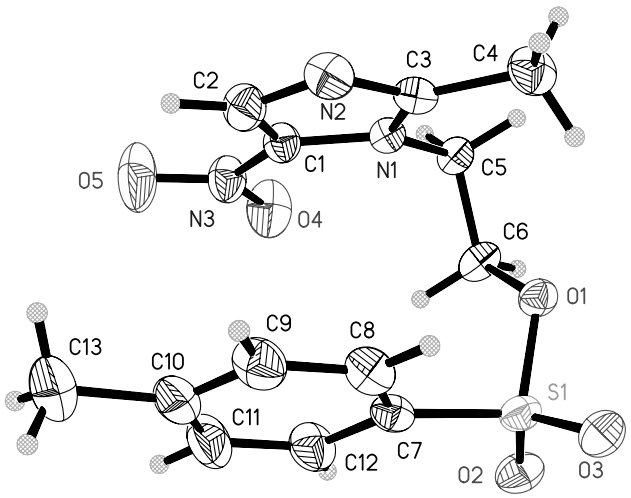


Fig. 1 Molecular structure of 1. The displacement ellipsoids are drawn at the probability level

C2) and 0.022 Å (for C3) from the plane of the ring of compound **2**, 0.144 (for C4) and 0.004 Å (for C6) of compound **3**, 0.229 (C5) and 0.027 Å (C4) of compound **4**,

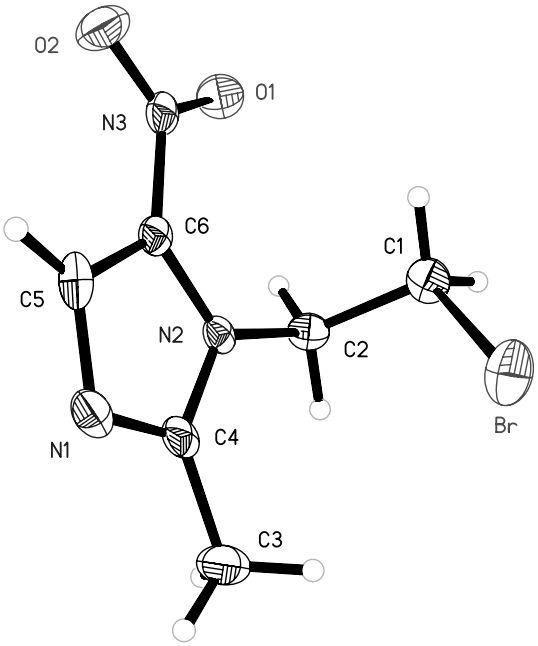


Fig. 2 Molecular structure of 2. The displacement ellipsoids are drawn at the probability level

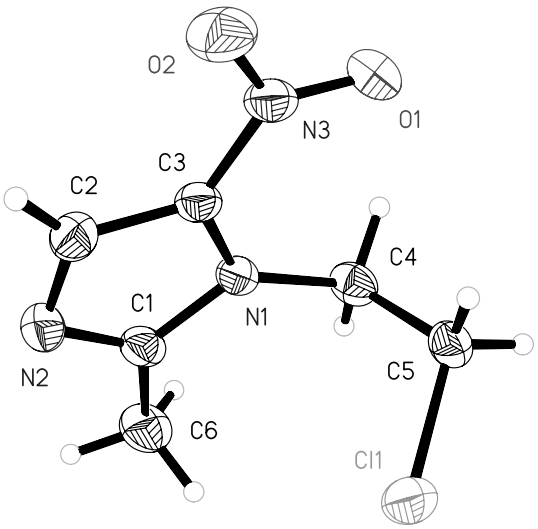


Fig. 3 Molecular structure of 3. The displacement ellipsoids are drawn at the probability level

respectively. The dihedral angle between the imidazole ring and the nitro plane is 6.6° for **2** (6.5° for **3**, 6.6° for **4**).

The selected bond distances and bond angles in **1–4** given in Table 2 are discussed as below. The crystal structures of compounds **2**, **3** and **4** are quite similar, they all consist of an imidazole ring, a nitro plane, and a halogen atom. As shown in table 1, the bond lengths of C–X are 1.897, 1.791 and 2.163 Å in **2**, **3**, and **4**, respectively, which well correspond to the size of the halogen atom and all conform to the normal value for the C–X bond. While for

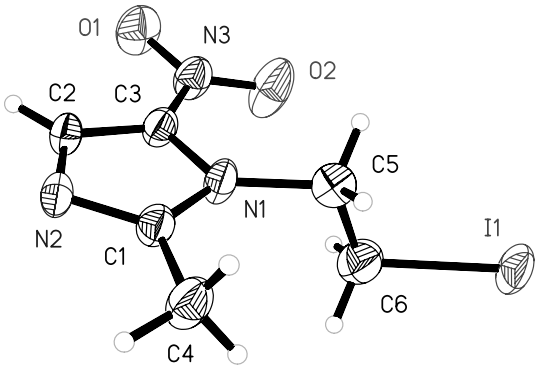


Fig. 4 Molecular structure of 4. The displacement ellipsoids are drawn at the probability level

Table 2 Selected bond lengths (Å) and angles (°) for **1–4**

MET-OTs (1)			
C(2)–C(1)	1.357	C(1)–N(3)	1.407
C(1)–N(1)	1.381	C(3)–N(1)	1.353
C(2)–N(2)	1.348	C(5)–N(1)	1.469
C(3)–N(2)	1.318	C(6)–O(1)	1.462
O(1)–C(6)–C(5)–N(1)	−63.35	O(5)–N(3)–C(1)–N(1)	5.3
O(4)–N(3)–C(1)–N(1)	2.9		
MET-Br (2)			
C(5)–C(6)	1.368	C(4)–N(2)	1.348
C(5)–N(1)	1.355	C(6)–N(3)	1.413
C(6)–N(2)	1.383	C(2)–N(2)	1.455
C(4)–N(1)	1.327	C(1)–Br(1)	1.897
Br(1)–C(1)–C(2)–N(2)	59.2	O(1)–N(3)–C(6)–N(2)	4.8
O(2)–N(3)–C(6)–N(2)	−176.1		
MET-Cl (3)			
C(2)–C(3)	1.366	C(1)–N(1)	1.365
C(2)–N(2)	1.343	C(3)–N(3)	1.407
C(3)–N(1)	1.382	C(4)–N(1)	1.465
C(1)–N(2)	1.339	C(5)–Cl(1)	1.791
Cl(1)–C(5)–C(4)–N(1)	60.3	O(1)–N(3)–C(3)–N(1)	4.4
O(2)–N(3)–C(3)–N(1)	−175.94		
MET-I (4)			
C(2)–C(3)	1.348	C(1)–N(1)	1.358
C(2)–N(2)	1.345	C(3)–N(3)	1.414
C(3)–N(1)	1.385	C(5)–N(1)	1.516
C(1)–N(2)	1.334	C(6)–I(1)	2.163
I(1)–C(6)–C(5)–N(1)	−179.9	O(2)–N(3)–C(3)–N(1)	8.3
O(1)–N(3)–C(3)–N(1)	−171.4		

compound **1**, the bond length of C–O is 1.462 Å, which is the shortest because the size of O atom is smaller than that of the halogen atoms.

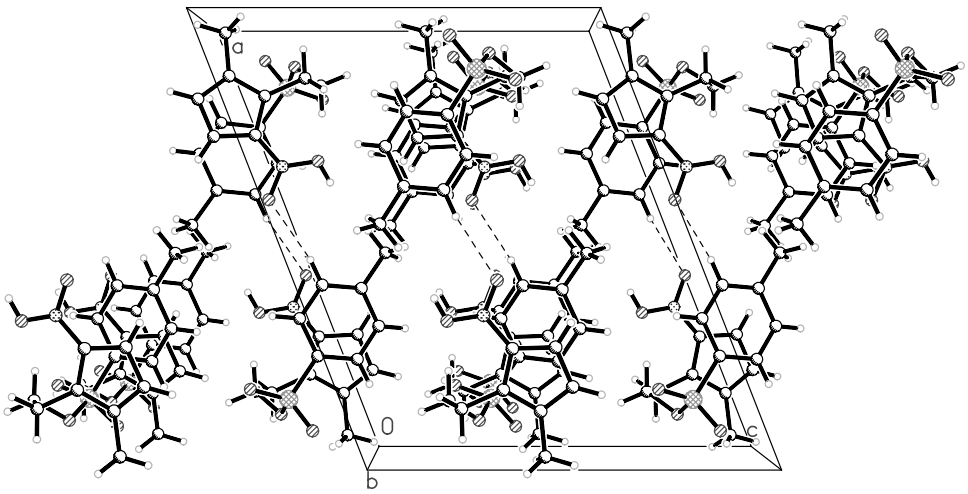


Fig. 5 The packing structure of compound **1** along the b-axis showing hydrogen bonds

In the imidazole ring, the double bond lengths of C–N are 1.318, 1.327, 1.339, and 1.334 Å; the double bond lengths of C–C are 1.357, 1.368, 1.366, and 1.348 Å in **1**, **2**, **3**, and **4**, respectively, typical of double bonds. The packing structure of compound **1** along the b-axis showing hydrogen bonds was listed in Fig. 5.

For compound **1**, the O(1)–C(6)–C(5)–N(1), O(5)–N(3)–C(1)–N(1), and O(4)–N(3)–C(1)–N(1) torsion angles in the central part of the molecule are −63.35, 5.3, and 2.9°, respectively. Correspondingly, they are 59.2, 4.8, −176.1; 60.3, 4.4, −175.94; −179.9, 8.3, −171.4° in compounds **2**, **3**, and **4**, respectively, and these are all in agreement with the values found in the analogous compound. Though the compounds **2**, **3** and **4** are iso-structures, the compound **4** crystallizes in a different crystal system with different set of unit cell parameters, while the isomorphism is clearly seen in **2** and **3** compounds. One of the causing factors is that N–C–C–X torsion angle. In the **2** and **3** compounds it is the gauche+ range whereas it got transformed to trans region in **4**.

Supplementary Material

Crystallographic data have been deposited with the Cambridge Crystallographic Data Centre (CCDC) (E-mail: deposit@ccdc.cam.ac.uk) as supplementary material and the CCDC numbers are 609616 (MET-Br) & 609617 (MET-OTs).

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Short communication

Imidazole derivatives as possible microbicides with dual protection☆

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ABSTRACT

Twenty seven derivatives (**2**–**28**) of 2-(2-methyl-5-nitro-1*H*-imidazol-1-yl)ethanol were synthesized and evaluated for anti-*trichomonas*, spermicidal and antifungal activities. Twenty six compounds were active against *Trichomonas vaginalis* at MIC ranging from 1–42 μM and seven compounds (**9**,**18**,**19**,**22**,**24**,**26**,**28**) immobilized 100% human spermatozoa at 1% concentration (w/v). Twenty three compounds (**2**,**3**,**5**,**8**–**26**,**28**) exhibited antifungal activity at 25–50 μg/mL concentration. Seven compounds (**9**,**18**,**19**,**22**,**24**,**26**,**28**) showed significant anti-*trichomonas* and spermicidal activities and also exhibited mild antifungal activity. All the compounds were highly safe towards human cervical cell line (HeLa) as shown by the cell-viability assay of HeLa cells at 200 μg/mL concentration, whereas nonoxynol-9 (N-9, the marketed spermicidal microbicide) was highly cytotoxic. Therefore, it may be concluded that introduction of the pharmacophore responsible for spermicidal activity into a proven anti-*trichomonas* structure may lead to a potent dual function microbicide better and safer than N-9.

1. Introduction

The epidemics of AIDS and unwanted pregnancies appear to thrive in the presence of overpopulation [1], poverty and other sexually transmitted diseases (STDs) [2–4]. It is now well established that trichomoniasis [5] (the most prevalent, non-viral STD) significantly increases the vulnerability to HIV [6,7] and therefore controlling trichomoniasis alone could significantly reduce the incidence of new HIV infections. On the other hand, high number of unintended pregnancies [8] may also indicate the need for newer, cost-effective, safe and convenient contraceptives. A recent survey [9] has shown that most women would prefer a product capable of preventing HIV, pregnancy and STIs and almost half of respondents would use contraceptive microbicides as a dual protection method. Therefore, efforts are being undertaken to develop novel, dually-active microbicidal agents [10–17]. Also since microbicides lacking contraceptive activity may not be used with the required compliance, especially during the "most vulnerable" sexual contacts where the immediate worry of an unwanted pregnancy often overwhelms the otherwise bigger fret of STD and/or HIV acquisition [11], it would be worthwhile to develop dually active agents. The healthy human vagina is firmly resistant to HIV infection [18], still ~5 million new patients are added annually to ~40 million living with HIV, half of which are women [18]. This indicates, (a) high prevalence of HIV in heterosexual contacts, and (b), increase in number of women with compromised vaginal resistance caused by vaginally applied chemical products and/or STD pathogens. Currently all commercially available spermicidal microbicides [19] have detergent ingredients that disrupt cell membranes. The most widely used vaginal spermicide, nonoxynol-9 (N-9) has been shown to damage the cervicovaginal epithelium because of its membrane-disruptive properties, causing an acute inflammatory tissue response, altered vaginal microflora, and enhanced risk of opportunistic infections in the genitourinary tract. Such opportunistic infections are known to enhance the susceptibility of the ectocervical epithelium and the endocervical mucosa to HIV infection. Hence, despite its ability to inactivate HIV *in vitro*, the reported failure [20] of N-9 to prevent heterosexual vaginal transmission of HIV in clinical settings has prompted the search for new female-controlled, non-detergent, topical vaginal spermicidal microbicides that are more effective as well as safer than N-9 [17]. Thus, a challenge is thrown open to chemists to design molecules with spermicidal and anti-HIV/STI activities. Consequently, it was thus thought worthwhile to introduce the pharmacophore responsible for spermicidal activity into a proven structure with anti-STI potential (and vice versa).

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Previously, some attempts have been made to modify metronidazole structure with a view to improve microbicidal activities [21–23]. Accordingly, the authors were prompted to carry out modifications in the structure of 2-(2-methyl-5-nitro-1*H*-imidazol-1-yl)ethanol (Metronidazole, 1, Scheme 1, the currently used drug for vaginal trichomoniasis [24]) with a view to introduce spermicidal activity. The pharmacophores imparting spermicidal activity have been reported to be dithiocarbamates [25], disulfide esters [14], benzenepropanamine [26], selective serotonin reuptake inhibitor (SSRI) antidepressants [27], thiourea derivatives [13], acrylophenones [28] etc. It was planned to derivatize at hydroxyl group of the side chain (Gen. Str I, Fig. 1), to introduce alkylamino ester (Gen. Str II, Fig. 1) and dithiocarbamaoyl group (Gen. Str III, Fig. 1) at position-2 of side chain. The compounds (**2**–**28**) synthesised were screened for spermicidal, anti-*trichomonas* and antifungal activities and also their safety was evaluated against human cervical (*HeLa*) cell line.

2. Results and discussion

2.1. Chemistry

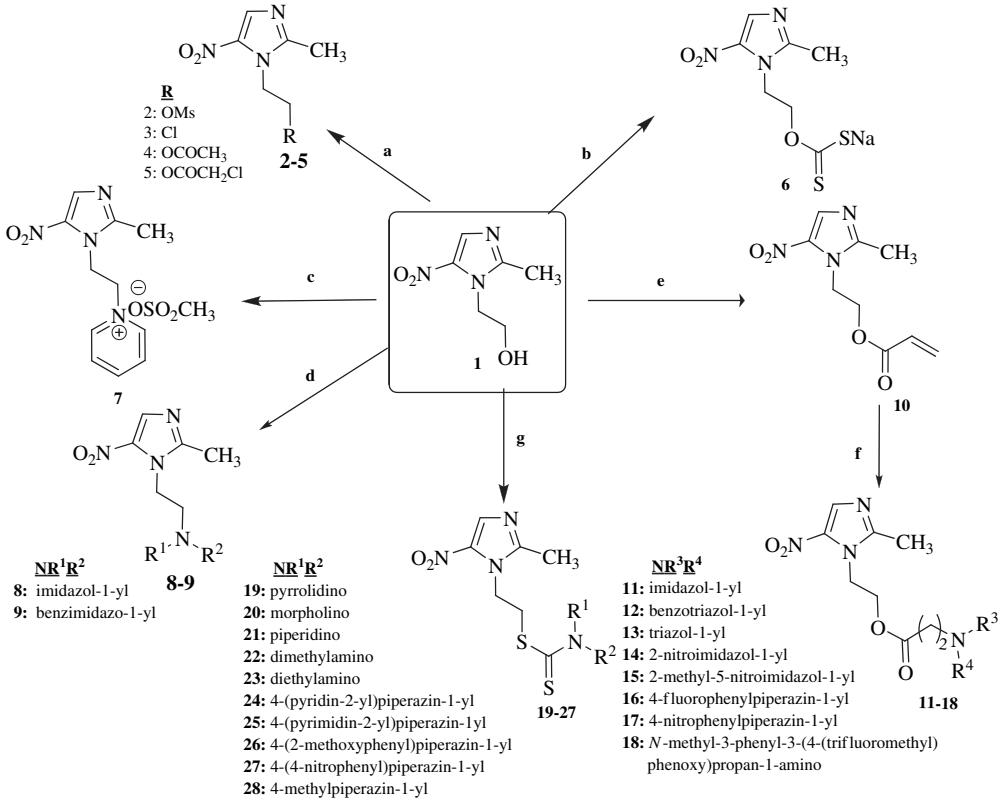
Compounds (**2**–**10**, Scheme 1) were synthesized by the reactions of appropriate reagents (**a**–**e**, Scheme 1) on hydroxyl group of 2-(2-methyl-5-nitro-1*H*-imidazol-1-yl)ethanol (**1**, Scheme 1). Compounds (**11**–**18**, Scheme 1) were prepared from 2-(2-methyl-5-nitro-1*H*-imidazol-1-yl)ethyl acrylate (**10**, Scheme 1) on addition with substituted azoles, substituted piperazines or *N*-methyl-3-phenyl-3-(4-(trifluoromethyl)phenoxy)propan-1-amine in the presence of triethylamine. Compounds (**19**–**28**, Scheme 1) were synthesized by the reaction of compound **2** with sodium salt of substituted carbodithioic acid in acetonitrile under reflux.

2.2. Biology

The compounds synthesized (**2**–**28**) were evaluated for spermicidal, anti-*trichomonas* (Table 1) and antifungal activities (Table 2). All the compounds were active against *Trichomonas vaginalis* at MIC ranging from 1.0–111.0 μM (Table 1) whereas the standard drug Metronidazole was active at 11.7 μM. Seven compounds (**9**,**18**,**19**,**22**,**24**,**26**,**28**) immobilized 100% human spermatozoa at 1% concentration (w/v) (Table 1). Twenty three compounds (**2**,**3**,**5**,**8**–**26**,**28**) exhibited antifungal activity at 50 μg/mL concentration (Table 2). Metronidazole, and all of the compounds (except 5) were highly safe towards human cervical cell line (HeLa) as shown by the cell-viability assay of HeLa cells at 200 μg/mL concentration, whereas N-9 was highly cytotoxic (Fig. 2).

The results indicated that on functionalization (**2**–**10**) at hydroxyl group in 2-(2-methyl-5-nitro-1*H*-imidazol-1-yl)ethanol (**1**) the anti-*trichomonas* activity was retained, and high spermicidal activity (100% immobilization) in compound **9** suggested that we have been able to introduce the dual activity as metronidazole (**1**) showed no effect on sperm motility (Table 1). On the other hand, compounds **2**–**8** exhibited moderate to appreciable anti-trichomonas activity but their spermicidal activity was negligible. However, 5-nitroimidazoles bearing an arylsulfonylmethyl group [21] and N-acetamide(sulfonamide)-2-methyl-4-nitro-1*H*-imidazoles [22] have been reported to exhibit substantial activity against *T. vaginalis*.

With a view to further enhance the spermicidal action in 2-(2-methyl-5-nitro-1*H*-imidazol-1-yl)ethanol framework, alkylamino side chain (**11**–**18**) and dithiocarbamoyl moieties (**19**–**28**) were introduced at hydroxyl function. The compounds with alkylamino side chain showed high anti-*trichomonas* activity. Compounds (**16**,**17**) were seven to eight times more active than metronidazole



Scheme 1. Reagents and conditions; a: CH_3SO_2Cl, Et_3N, CH_2Cl_2, 0–5 °C/ $SOCl_2$, $CHCl_3$(dry), reflux/ $(CH_3CO)_2O$, Et_3N, CH_2Cl_2, DMAP, 0–5 °C/ $ClCH_2COCl$, Et_3N, CH_2Cl_2, 0–5 °C; b: CS_2, NaOH, MeOH, rt; c:(i) CH_3SO_2Cl, Et_3N, CH_2Cl_2, 0–5 °C; (ii) Py, 110-115°C; d: (i) CH_3SO_2Cl, Et_3N, CH_2Cl_2, 0–5 °C; (ii) NR^1R^2, Et_3N, toluene, reflux; e: $ClCOCH_2CH_2Cl$, Et_3N, CH_2Cl_2, 0–5 °C; f: NR^3R^4, Et_3N, CH_2Cl_2/$ClCH_2CH_2Cl$; g:(i) CH_3SO_2Cl, Et_3N, CH_2Cl_2, 0–5 °C; (ii) $R^1R^2NC{=}SSNa$, CH_3CN, reflux.

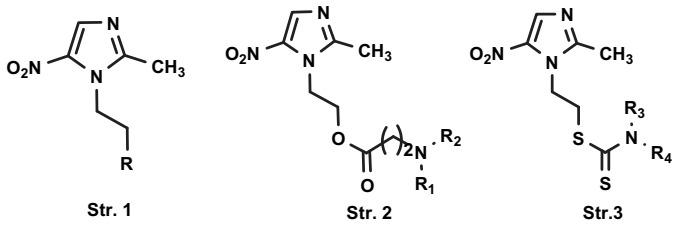
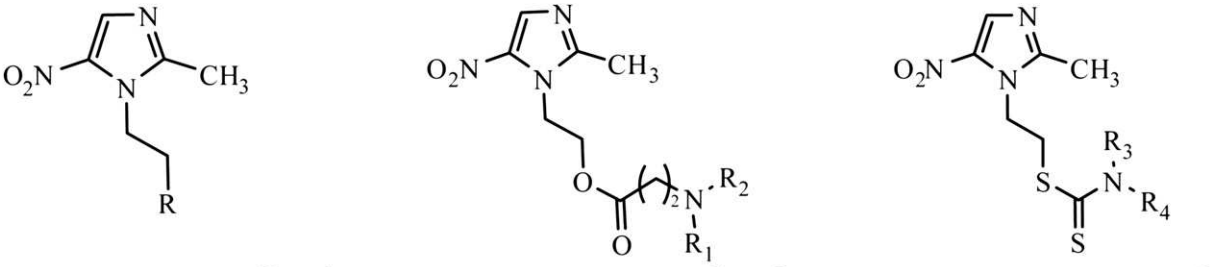


Fig. 1. General Structures of the compounds synthesized.

and compound **18** immobilized 100% sperms at 1% concentration. The addition of dithiocarbamoyl moiety further enhanced both the activities. Three compounds **21**–**23** were ten to eleven times, compound **20** six times and compound **27** was about four times as active as metronidazole and four compounds (**19**,**24**,**26**,**28**) had MIC significantly better than metronidazole. Moreover, five compounds (**19**,**22**,**24**,**26**,**28**) also immobilized 100% human spermatozoa and therefore exhibited dual activity. Compound **22** showed remarkable anti-*trichomonas* and spermicidal activities and also exhibited mild antifungal activity. The notable activity in seven compounds (**9**,**18**,**19**,**22**,**24**,**26**,**28**) may possibly be attributed to their high sulfhydryl affinity [14] resulting in interactions between sulfhydryl group present on sperm membrane with benzimidazolyl side-chain, benzenepropanamine [26] and dithiocarbamoyl group [16], respectively.

Therefore, it may be concluded that introduction of the pharmacophore responsible for spermicidal activity into a proven anti-*trichomonas* structure may lead to a potent dual function spermicide, which can be better and safer than N-9.

Table 1
In-vitro Spermicidal activity and Anti-*Trichomonas* activity of the compounds (**2**–**28**) against human spermatozoa and *Trichomonas vaginalis*.



Compounds (str. 1)	R	Spermicidal activity (percent sperm motility of control at 1.0% concentration)	Anti-*Trichomonas* activity (μM) (MIC ± SE)
2	OMs	94.12	5.02 ± 0.89
3	Cl	58.82	6.60 ± 0.44
4	OAc	82.35	23.47 ± 4.14
5	$OCOCH_2Cl$	98.37	10.10 ± 1.78
6	OC = SSNa	90.24	111.52 ± 4.96
7	Pyridinium methanesulfonate	85.09	9.53 ± 1.02
8	imidazol-1-yl	92.36	14.14 ± 1.51
9	benzimidazol-1-yl	0	4.61 ± 0.81
10	acrylate	70.58824	42.22 ± 2.96
Compounds (str. 2)	NR_1R_2		
11	Imidazol-1-yl	100	10.67 ± 3.01
12	Benzotrizol-1-yl	95.734	27.62 ± 0.32
13	Triazol-1-yl	100	21.26 ± 0.57
14	2-nitroimidazol-1-yl	100	9.25 ± 0.99
15	2-methyl-5-nitroimidazol-1-yl	88.23529	20.60 ± 1.71
16	4-fluorophenylpiperazin-1-yl	88.23529	1.54 ± 0.82
17	4-nitrophenylpiperazin-1-yl	82.35294	1.45 ± 0.77
18	N-methyl-3-phenyl-3-(4-(trifluoromethyl) phenoxy)propan-1-amino	0	11.70 ± 0.21
Compounds (str. 3)	NR_3R_4		
19[a]	pyrrolidino	0	8.33 ± 1.47
20	morpholino	70.58824	1.98 ± 0.35
21	piperidino	70.58824	1.00 ± 0.18
22[a]	dimethylamino	0	1.14 ± 0.20
23	diethylamino	100	1.03 ± 0.18
24[a]	4-(pyridin-2-yl)piperazin-1-yl	0	6.38 ± 0.43
25	4-(pyrimidin-2-yl)piperazin-1yl	94.11765	25.45 ± 2.94
26[a]	4-(2-methoxyphenyl)piperazin-1-yl	0	5.94 ± 0.69
27	4-(4-nitrophenyl)piperazin-1-yl	41.17647	2.87 ± 0.51
28	4-methylpiperazin-1-yl	0	7.60 ± 0.33
N-9	–	0	60.78 ± 4.05
Metronidazole	–	100	11.70 ± 1.46
Vehicle (**Control**)	–	100	Inactive

[a] These compounds were tested as D-(-)tartrate salt for spermicidal activity.

Table 2
Antifungal activity against six different strains of fungi.

Compound	Antifungal activity (MIC in μg/mL)					
	1	2	3	4	5	6
2	>50	>50	50	>50	>50	>50
3	>50	50	50	>50	>50	>50
4	>50	>50	>50	>50	>50	>50
5	>50	>50	>50	25	>50	>50
6	>50	>50	>50	>50	>50	>50
7	>50	>50	>50	>50	>50	>50
8	25	>50	50	>50	>50	>50
9	>50	>50	50	>50	>50	>50
10	>50	50	50	50	>50	>50
11	>50	>50	>50	50	>50	>50
12	>50	>50	>50	50	>50	>50
13	>50	>50	>50	50	>50	>50
14	>50	>50	>50	50	>50	>50
15	>50	>50	>50	50	>50	>50
16	>50	>50	>50	50	>50	>50
17	>50	>50	>50	50	>50	>50
18	>50	>50	>50	50	>50	>50
19	50	50	25	50	>50	>50
20	>50	>50	50	50	>50	>50
21	>50	>50	25	50	>50	>50
22	>50	>50	>50	50	>50	>50
23	50	50	25	25	>50	>50
24	>50	>50	50	50	>50	>50
25	>50	>50	>50	50	>50	>50
26	>50	>50	50	25	>50	>50
27	>50	>50	>50	>50	>50	>50
28	>50	>50	50	50	>50	>50
N-9	>50	–	–	–	–	50
Fluconazole	0.5	1.0	2.0	1.0	2.0	1.0

1: *Candida albicans*; 2: *Cryptococcus neoformans*; 3: *Sporothrix schenckii*; 4: *Trichophyton mentagrophytes*; 5: *Aspergillus fumigatus*; 6: *Candida parapsilosis*.

3. Experimental section

3.1. Chemistry

Melting points were determined in open capillary tubes on an electrically heated block and are uncorrected. IR spectra (ν_{max} in cm^{-1}) of the compounds were recorded on Perkin Elmer's FT-IR RX1 PC spectrophotometer. ^1H NMR & ^{13}C NMR spectra were recorded on Bruker Supercon Magnet Avance DPX-200/DRX-300 spectrometers (operating at 200 and 300 MHz respectively for ^1H; 50 and 75 MHz respectively for ^{13}C) in deuterated solvents with TMS as internal reference (chemical shifts in δ ppm, J in Hz.). Electronspray Ionisation Mass spectra (ESI-MS) were recorded on a Micromass Quattro II triple quadruple mass spectrometer. Elemental analyses were performed on Carlo Erba EA-1108 micro analyzer/Vario EL-III C H N S analyzer. All compounds were analyzed of C, H, N and the results obtained were within ±0.4% of calculated values. The reaction progress was routinely monitored by thin layer chromatography (TLC) on precoated alumina/silica gel plates (Aldrich). Column chromatography was performed over Merck silica gel (60–120 mesh). All chemicals and solvents were procured from Sigma-Aldrich/Merck India Ltd. 1-dialkylamino carbodithioic acid sodium salts [25] were prepared by known procedures.

3.1.1. 2-(2-methyl-5-nitro-1H-imidazol-1-yl)ethyl methanesulfonate (**2**)

Into a ice-cooled solution of 2-(2-methyl-5-nitro-1*H*-imidazol-1-yl)ethanol (**1**, 0.011 mol) and triethylamine (0.017 mol) in dry dichloromethane was added methane sulfonyl chloride (0.017 mol) drop wise under stirring. The reaction mixture was further stirred at 0–5 °C for 2 h. The separated thick material was filtered and washed with water (15 mL × 3) gave a cream coloured solid. The solid was crystalized from acetonitrile/ethyl acetate (1:1, v/v). Yield 80%; Mp 139–140 °C; IR (KBr): 3024, 2934, 1526, 1260, 745 cm^{-1}; ESI-MS: m/z 250 ($M^+ + 1$); ^1H NMR (300 MHz, DMSO-d_6, δ ppm): δ 8.05 (s, 1H), 4.65 (t, 2H, $j = 4.7$ Hz), 4.55 (t, 2H, $j = 4.6$ Hz), 3.14 (s, 3H), 2.46 (s, 3H); Anal. Calcd. for $C_7H_{11}N_3O_5S$ (249): C, 33.73; H, 4.45; N, 16.86; Found: C, 33.51; H, 4.25; N, 16.53.

3.1.2. 1-(2-chloroethyl)-2-methyl-5-nitro-1H-imidazole (**3**)

Into a ice-cooled solution of 2-(2-methyl-5-nitro-1*H*-imidazol-1-yl)ethanol (**1**, 0.003 mol) in dry chloroform (20 mL) was added thionyl chloride (0.004 mol) dropwise under stirring. A sticky solid separated out which was dissolved on heating the mixture to 65 °C in an oil bath. The reaction mixture was further heated at 65–70 °C for 3 h. Excess thionyl chloride was removed azeotropically. The residue was taken into ethyl acetate (30 mL) and washed with water (10 mL × 3). Organic layer was dried over sodium sulfate and concentrated under reduce pressure to provide a brown solid. Yield 85%; Mp 78–79 °C; IR (KBr): 3132, 2976, 1531, 1365, 1263, 756 cm^{-1}; ESI-MS: m/z 190 ($M^+ + 1$), 192 ($M^+ + 3$); ^1H NMR (300 MHz, $CDCl_3$, δ ppm): δ 7.91 (s, 1H), 4.61 (t, 2H, $j = 5.6$ Hz), 3.87 (t, 2H, $j = 5.6$ Hz), 2.54 (s, 3H); Anal. Calcd.: for $C_6H_8ClN_3O_2$ (189): C, 38.01; H, 4.25; N, 22.16; Found: C, 37.93; H, 4.16; N, 22.01.

3.1.3. Acetic acid 2-(2-methyl-5-nitro-1H-imidazol-1-yl) ethyl ester (**4**)

In to an ice-cooled solution of 2-(2-methyl-5-nitro-1*H*-imidazol-1-yl)ethanol (**1**, 0.015 mol) in dry dichloromethane (10 mL) was added triethylamine (0.022 mol) and dimethylaminopyridine

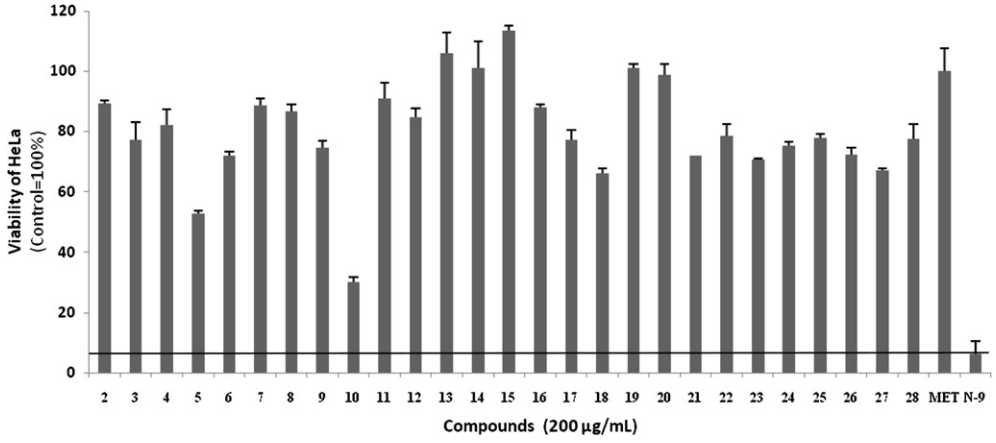


Fig. 2. The viability of HeLa cells after 24 h incubation in 200 μg/mL of test compounds (Mean ± SE of 3 estimations). MET (metronidazole); N-9 (nonoxynol-9).

(10 mol%) under stirring. Acetic anhydride (0.003 mol) was added dropwise and the reaction mixture was further stirred for 1 h (0–5 °C). The reaction mixture was concentrated under reduced pressure, diluted with water (15 mL) and extracted with ethyl acetate (15 mL × 3). The combined organic phase was washed with water (10 mL × 3) and dried over sodium sulfate. Sodium sulfate was filtered off and washed with ethyl acetate (5 mL × 2). The combined ethyl acetate layer was concentrated under reduce pressure to give a light brown solid. Yield 88%; Mp 61–62 °C; IR (KBr): 3124, 2963, 1741, 1528, 1263, 744 cm^{-1}; ESI-MS: *m/z* 213 (M^+), 214 ($M^+ + 1$); ^1H NMR (300 MHz, $CDCl_3$, δ ppm): δ 7.92 (s, 1H); 4.55 (t, 2H, *j* = 5.2 Hz), 4.37 (t, 2H, *j* = 5.1 Hz), 2.47 (s, 3H), 1.99 (s, 3H); Anal. Calcd. for $C_8H_{11}N_3O_4$ (213): C, 45.07; H, 5.20; N, 19.71; Found: C, 45.12; H, 5.39; N, 19.61.

3.1.4. Chloro-acetic acid 2-(2-methyl-5-nitro-1H-imidazol-1-yl) ethyl ester (**5**)

Into a ice-cooled solution of 2-(2-methyl-5-nitro-1*H*-imidazol-1-yl)ethanol (**1**, 0.0011 mol) and triethylamine (0.0022 mol) in dichloromethane (10 mL) was added chloroacetyl chloride (0.0022 mol) dropwise under stirring. The reaction mixture was further stirred at 0–5 °C for 30 min. Dichloromethane was distilled off and the residue was taken in ethyl acetate (20 mL). The ethyl acetate layer was washed with water (10 mL × 3) and dried over sodium sulfate. Sodium sulfate was filtered off and washed with ethyl acetate (5 mL × 2). The filtrate was concentrated to give a brown semisolid. Yield 71%; IR (KBr): 3021, 1747, 1534, 1364, 1262, 756 cm^{-1}; ESI-MS: *m/z* 248 ($M^+ + 1$), 250 ($M^+ + 3$); ^1H NMR (300 MHz, $CDCl_3$, δ ppm): δ 7.95 (s, 1H), 4.62 (t, 2H, *j* = 4.9 Hz), 4.52 (t, 2H, *j* = 4.9 Hz), 4.01 (s, 2H), 2.53 (s, 3H); Anal. Calcd. for $C_8H_{10}ClN_3O_4$ (247): C, 38.80; H, 4.07; N, 16.97; Found: C, 38.52; H, 4.15; N, 17.13.

3.1.5. Sodium O-2-(2-methyl-5-nitro-1H-imidazol-1-yl)ethyl carbonodithioate (**6**)

The title compound was prepared by the known procedure [29]. To a ice-cooled solution of sodium hydroxide (0.0028 mol, 0.287 g) in methanol (10 mL) was added 2-(2-methyl-5-nitro-1*H*-imidazol-1-yl)ethanol (**1**, 0.0018 mol) in small portions under stirring. Carbon disulfide was added dropwise at 0–5 °C and the reaction mixture was further stirred for 24 h at room temperature, during the course of reaction pH maintained basic (10–12). Methanol was distilled off and crude product was taken in acetone (10 mL) to give a precipitate. Yellow solid was filtered off and dried in vacuum desiccator. Yield 77%; Mp 250 °d; IR (KBr): 1640, 1384, 1272, 674 cm^{-1}; ^1H NMR (300 MHz, D_2O, δ ppm): δ 7.31 (s, 1H), 3.97 (t, 2H, *j* = 5.2 Hz), 3.75 (t, 2H, *j* = 5.1 Hz), 2.26 (s, 3H); Anal. Calcd. for $C_7H_8N_3NaO_3S_2$ (269): C, 31.22; H, 2.99; N, 15.60; Found: C, 31.39; H, 3.17; N, 15.91.

3.1.6. 1-[2-(2-methyl-5-nitro-1H-imidazol-1-yl)-ethyl]pyridinium methanesulfonate (**7**)

2-(2-methyl-5-nitro-1*H*-imidazol-1-yl)ethyl methanesulfonate (**2**, 0.0006 mol) was added into excess of pyridine (2 mL) and the reaction mixture was heated at reflux under stirring for 24 h. Pyridine was distilled off under reduced pressure. The residual brown solid was washed with ethyl acetate/hexane mixture (1:1; 10 mL × 2). Yield 82%; Mp 145–146 °C; IR (KBr): 3444, 2929, 1742, 1650, 1187, 778 cm^{-1}; ^1H NMR (300 MHz, D_2O, δ ppm): δ 8.48 (*d*, 2H, *j* = 5.9 Hz), 8.36–8.31 (m, 1H), 7.82 (s, 1H), 7.80–7.77 (m, 2H), 4.82 (t, 2H, *j* = 5.5 Hz), 4.69 (t, 2H, *j* = 5.4 Hz), 2.48 (s, 3H), 1.88 (s, 3H); Anal. Calcd. for $C_{12}H_{16}N_4O_5S$ (328): C, 43.90; H, 4.91; N, 17.06; Found: C, 43.53; H, 5.21; N, 17.31.

3.1.7. Synthesis of 1-(2-(2-methyl-5-nitro-1H-imidazol-1-yl)ethyl) 1H-substituted azoles (**8**,**9**; Scheme 1)

To a solution of 2-(2-methyl-5-nitro-1*H*-imidazol-1-yl)ethyl methanesulfonate (1 eq) and triethylamine (1.5 eq) in toluene (10 mL) was added imidazole/benzimidazole (1 eq) portionwise at room temperature. The reaction mixture was heated at 110–120 °C for 6 h under stirring in an oil bath. Toluene was distilled off under reduced pressure and the crude material was taken in chloroform (20 mL). The chloroform layer was washed with water (10 mL × 3) and dried over sodium sulfate. Sodium sulfate was filtered off and washed with chloroform (5 mL × 2). The filtrate was concentrated under reduced pressure. The crude material was purified by column chromatography over silica gel (60–120 mesh) with chloroform/hexane as an eluent.

3.1.7.1. 1-(2-(1H-imidazol-1-yl)ethyl) 2-methyl-5-nitro-1H-imidazole (**8**).

Light red solid; Yield 63%; Mp 158–159 °C; IR (KBr): 3032, 2964, 1531, 1259, 736 cm^{-1}; ESI-MS: *m/z* 222 ($M^+ + 1$), 154; ^1H NMR (300 MHz, $CDCl_3$, δ ppm): δ 7.97 (s, 1H), 7.26 (s, 1H), 7.03 (s, 1H), 6.60 (s, 1H), 4.54 (t, 2H, *j* = 5.4 Hz), 4.36 (t, 2H, *j* = 5.4 Hz), 1.85 (s, 3H); Anal. Calcd. for $C_9H_{11}N_5O_2$ (221): C, 48.86; H, 5.01; N, 31.66; Found: C, 48.58; H, 5.34; N, 31.82.

3.1.7.2. 1-(2-(2-methyl-5-nitro-1H-imidazol-1-yl)ethyl) 1H-benzimidazole (**9**).

Light yellow solid; Yield 67%; Mp 210–211 °C; I.R.(KBr): 2922, 1519, 1257, 760 cm^{-1}; ESI-MS: *m/z* 272 ($M^+ + 1$); ^1H NMR (300 MHz, $CDCl_3$, δ ppm): δ 8.00 (s, 1H), 7.83–7.79 (m, 1H), 7.64 (s, 1H), 7.33–7.30 (m, 2H), 7.23–7.20 (m, 1H), 4.69–4.61 (m, 4H), 1.70 (s, 3H); Anal. Calcd. for $C_{13}H_{13}N_5O_2$ (271): C, 57.56; H, 4.83; N, 25.82; Found: C, 57.87; H, 4.96; N, 25.49.

3.1.8. Acrylic acid 2-(2-methyl-5-nitro-1H-imidazol-1-yl)e thyl ester (**10**)

To a ice-cooled solution of 2-(2-methyl-5-nitro-1*H*-imidazol-1-yl)ethanol (**1**, 0.023 mol) and triethylamine (0.035 mol) in dry dichloromethane (70 mL) was added a solution of 1,3-dichloropropanone (0.046 mol) in chloroform (10 mL) under stirring in 2 h. The reaction mixture was allowed to attain room temperature in 1 h under stirring. The reaction mixture was further stirred for 3 h at room temperature. The reaction mixture was concentrated and then diluted with chloroform (50 mL) and water (20 mL). The two layers were stirred for 30 min and then separated. Organic layer was washed with water (15 mL × 3) and dried over sodium sulfate. Sodium sulfate was filtered off and washed with chloroform (5 mL × 2) and the filtrate was concentrated under reduce pressure. A yellowish solid was formed. Yield 79%; Mp 42–43 °C; IR (KBr): 3123, 2957, 1721, 1529, 1369, 1264, 742 cm^{-1}; ESI-MS: *m/z* 226 ($M^+ + 1$); ^1H NMR (300 MHz, $CDCl_3$, δ ppm): δ 7.92 (s, 1H), 6.34 (d, 1H, *j* = 15.8 Hz), 6.07–5.97 (m, 1H), 5.85 (d, 1H, *j* = 9.0 Hz), 4.61–4.58 (m, 2H), 4.49–4.48 (m, 2H), 2.46 (s, 3H); Anal. Calcd. for $C_9H_{11}N_3O_4$ (225): C, 48.00; H, 4.92; N, 18.66; Found: C, 47.68; H, 5.24; N, 18.33.

3.1.9. Synthesis of 3-substituted-propionic acid 2-(2-methyl-5-nitro-1H-imidazol-1-yl)ethyl ester (**11**–**18**, Scheme 1)

To a solution of acrylic acid 2-(2-methyl-5-nitro-1*H*-imidazol-1-yl)-ethyl ester (**10**, 0.003 mol) in dichloromethane/dichloroethane (10 mL) was added substituted diazoles, substituted piperazines or *N*-methyl-3-phenyl-3-(4-(trifluoromethyl)phenoxy)propan-1-amine at room temperature under stirring. Reaction mixture was heated at reflux under stirring for 5–8 h in an oil bath. Solvent was distilled off and crude product was taken into ethyl acetate (20 mL). Organic layer was washed with water (10 mL × 3) and dried over sodium sulfate. Sodium sulfate was filtered off and washed with ethyl acetate (5 mL × 2) and the filtrate was concentrated under reduce pressure. Crude material was purified by column chromatography using chloroform/hexane or ethyl acetate/hexane as eluent to give the product.

*3.1.9.1. 3-Imidazol-1-yl-propionic acid 2-(2-methyl-5-nitro-1H-imidazol-1-yl)ethyl ester (**11**).* Light yellow solid; Yield 77%; Mp 81–82 °C; IR (KBr): 3019, 1744, 1534, 1428, 1263, 758 cm^{-1}; ESI-MS: *m/z* 294 ($M^+ + 1$); ^1H NMR (300 MHz, $CDCl_3$, δ ppm): δ 7.93 (s, 1H), 7.47 (s, 1H), 7.03 (s, 1H), 6.88 (s, 1H), 4.54 (t, 2H, *j* = 5.2 Hz), 4.40 (t, 2H, *j* = 5.1 Hz), 4.22 (t, 2H, *j* = 6.4 Hz), 2.71 (t, 2H, *j* = 6.4 Hz), 2.41 (s, 3H); Anal. Calcd. for $C_{12}H_{15}N_5O_4$ (293): C, 49.14; H, 5.16; N, 23.88; Found: C, 49.42; H, 5.29; N, 23.68.

*3.1.9.2. 3-Benzotriazol-1-yl-propionic acid 2-(2-methyl-5-nitro-1H-imidazol-1-yl)ethyl ester (**12**).* Light yellow solid; Yield 51%; Mp 94–95 °C; IR (KBr): 2969, 1742, 1530, 1364, 1263, 757 cm^{-1}; ESI-MS: *m/z* 345 ($M^+ + 1$); ^1H NMR (300 MHz, $CDCl_3$, δ ppm): δ 7.99 (d, 1H, *j* = 8.3 Hz), 7.81 (s, 1H), 7.54–7.43 (m, 2H), 7.35–7.30 (m, 1H), 4.81 (t, 2H, *j* = 6.5 Hz), 4.46 (t, 2H, *j* = 5.0 Hz), 4.34 (t, 2H, *j* = 4.9 Hz), 3.04 (t, 2H, *j* = 6.5 Hz), 2.34 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$, δ ppm): δ 170.0, 150.7, 145.9, 138.5, 133.2, 127.6, 124.0, 120.0, 109.4, 63.2, 44.9, 43.1, 34.0, 14.3; Anal. Calcd. for $C_{15}H_{16}N_6O_4$ (344): C, 52.32; H, 4.68; N, 24.41; Found: C, 52.09; H, 4.99; N, 24.25.

*3.1.9.3. 3-[1,2,4]Triazol-1-yl-propionic acid-2-(2-methyl-5-nitro-1H-imidazol-1-yl)ethyl ester (**13**).* Yellow solid; Yield 55%; Mp 57–58 °C; IR (KBr): 3018, 1743, 1533, 1365, 1265, 760 cm^{-1}; ESI-MS: *m/z* 295 ($M^+ + 1$); ^1H NMR (300 MHz, $CDCl_3$, δ ppm): δ 8.07 (s, 1H), 7.89 (s, 1H), 7.86 (s, 1H), 4.54 (t, 2H, *j* = 5.1 Hz), 4.41 (t, 4H, *j* = 5.6 Hz), 2.87 (t, 2H, *j* = 6.1 Hz), 2.42 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$, δ ppm): δ 169.8, 151.9, 150.3, 143.5, 138.0, 132.8, 62.8, 44.6, 44.3, 33.5, 14.0; Anal. Calcd. for $C_{11}H_{14}N_6O_4$ (294): C, 44.90; H, 4.80; N, 28.56; Found: C, 44.61; H, 4.98; N, 28.38.

*3.1.9.4. 3-(4-Nitro-1H-imidazol-1-yl)-propionic acid-2-(2-methyl-5-nitro-1H-imidazol-1-yl)ethyl ester (**14**).* Light yellow solid; Yield 68%; Mp 108–109 °C; IR (KBr): 3083, 2965, 1730, 1522, 1267, 744 cm^{-1}; ESI-MS: *m/z* 339 ($M^+ + 1$); ^1H NMR (300 MHz, $CDCl_3$, δ ppm): δ 8.02 (s, 1H), 7.92 (s, 1H), 7.58 (s, 1H), 4.61 (t, 2H, *j* = 4.9 Hz), 4.45 (t, 2H, *j* = 5.0 Hz), 4.33 (t, 2H, *j* = 6.0 Hz), 2.84 (t, 2H, *j* = 6.1 Hz), 2.48 (s, 3H); Anal. Calcd. for $C_{12}H_{14}N_6O_6$ (338): C, 42.61; H, 4.17; N, 24.84; Found: C, 42.38; H, 4.28; N, 24.69.

*3.1.9.5. 3-(2-Methyl-5-nitro-1H-imidazol-1-yl)-propionic acid 2-(2-methyl-5-nitro-1H-imidazol-1-yl)ethyl ester (**15**).* White solid; Yield 81%; Mp 207–208 °C; IR (KBr): 3055, 2899, 1742, 1506, 1260, 750 cm^{-1}; ESI-MS: *m/z* 353 ($M^+ + 1$); ^1H NMR (300 MHz, $CDCl_3$, δ ppm): δ 7.94 (s, 1H), 7.91 (s, 1H), 4.61 (t, 2H, *j* = 4.9 Hz), 4.45 (t, 2H, *j* = 5.0 Hz), 4.22 (t, 2H, *j* = 6.3 Hz), 2.83 (t, 2H, *j* = 6.3 Hz), 2.49 (s, 3H), 2.46 (s, 3H); Anal. Calcd. for $C_{13}H_{16}N_6O_6$ (352): C, 44.32; H, 4.58; N, 23.85; Found: C, 44.02; H, 4.68; N, 23.65.

*3.1.9.6. 2-(2-methyl-5-nitro-1H-imidazol-1-yl)ethyl-3-(4-(4–fluorophenyl)piperazin-1-yl)propanoate (**16**).* Yellow solid; Yield 81%; Mp 89–90 °C; IR (KBr): 2955, 1730, 1514, 1268, 744 cm^{-1}; ESI-MS: *m/z* 406 ($M^+ + 1$); ^1H NMR (200 MHz, $CDCl_3$, δ ppm): δ 7.89 (s, 1H), 6.95–6.77 (m, 4H), 4.57 (t, 2H, *j* = 5.2 Hz), 4.39 (t, 2H, *j* = 5.2 Hz), 3.06–3.01 (m, 4H), 2.69–2.61 (m, 2H), 2.58–2.53 (m, 4H), 2.49 (s, 3H), 2.46–2.42 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$, δ ppm): δ 171.3, 158.7, 155.5, 150.3, 147.8, 138.6, 132.8, 117.8, 117.7, 115.6, 115.3, 62.4, 53.2, 50.0, 44.8, 32.0, 14.2; Anal. Calcd. for $C_{19}H_{24}FN_5O_4$ (405): C, 56.29; H, 5.97; N, 17.27; Found: C, 56.60; H, 6.21; N, 17.22.

*3.1.9.7. 2-(2-methyl-5-nitro-1H-imidazol-1-yl)ethyl 3-(4-(4-nitrophenyl)piperazin-1-yl)propanoate (**17**).* Dark yellow solid; Yield 66%; Mp 115–116 °C; IR (KBr): 2926, 2823, 1738, 1596, 1526, 1248, 750 cm^{-1}; ESI-MS: *m/z* 433 ($M^+ + 1$); ^1H NMR (300 MHz, $CDCl_3$, δ ppm): δ 8.11 (d, 2H, *j* = 9.2 Hz), 7.92 (s, 1H), 6.81 (d, 2H, *j* = 9.3 Hz), 4.63 (t, 2H, *j* = 5.2 Hz), 4.43 (t, 2H, *j* = 5.2 Hz), 3.40–3.37 (m, 4H), 2.69 (t, 2H, *j* = 6.8 Hz), 2.59 (d, 4H, *j* = 4.9 Hz), 2.57 (s, 3H), 2.53–2.47 (m, 2H); Anal. Calcd. for $C_{19}H_{24}N_6O_6$ (432): C, 52.77; H, 5.59; N, 19.43; Found: C, 52.51; H, 5.81; N, 19.79.

*3.1.9.8. 2-(2-methyl-5-nitro-1H-imidazol-1-yl)ethyl 3-(methyl(3-phenyl-3-(4-(trifluoromethyl)phenoxy)propyl) amino)propanoate (**18**).* Light red semisolid; Yield 77%; IR (neat): 3020, 1740, 1616, 1529, 1326, 1216, 762 cm^{-1}; ESI-MS: *m/z* 535 ($M^+ + 1$); ^1H NMR (300 MHz, $CDCl_3$, δ ppm): δ 7.85 (s, 1H), 7.39 (d, 2H, *j* = 8.5 Hz), 7.30–7.22 (m, 5H), 6.84 (d, 2H, *j* = 8.5 Hz), 5.32–5.28 (m, 1H), 4.52–4.49 (m, 2H), 4.34–4.30 (m, 2H), 2.77–2.53 (m, 6H), 2.43 (s, 3H), 2.34 (s, 3H), 2.19–2.12 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$, δ ppm): δ 171.3, 160.6, 150.3, 141.0, 138.6, 132.8, 128.8, 127.8, 126.8, 126.75, 126.71, 126.1, 125.7, 123.1, 122.7, 122.5, 115.7, 78.0, 62.3, 53.4, 52.4, 44.8, 41.8, 36.1, 32.1, 14.1; Anal. Calcd. for $C_{26}H_{29}F_3N_4O_5$ (534): C, 58.42; H, 5.47; N, 10.48; Found: C, 58.73; H, 5.18; N, 10.76.

*3.1.10. 2-(2-methyl-5-nitro-1H-imidazol-1-yl)ethyl dialkylamino-1-carbodithioate (**19**–**28**, Scheme 1)*

To a solution of 2-(2-methyl-5-nitro-1*H*-imidazol-1-yl)ethyl methanesulfonate (**2**, 0.002 mol) in acetonitrile (20 mL) was added dialkylamino carbodithioic acid sodium salt (0.002 mol) at room temperature. The reaction mixture was heated at reflux in an oil bath under stirring for 6–9 h. The reaction mixture was concentrated and diluted with ethyl acetate (25 mL). The ethyl acetate layer was washed with water (10 mL × 3) and dried over sodium sulfate. Sodium sulfate was filtered off and washed with ethyl acetate (5 mL × 2). The filtrate was concentrated under reduce pressure to provide the crude product which was purified by column chromatography over silica gel with ethylacetate/hexane as eluent. The free base (1 eq) was dissolved in methanol (20 mL) and a solution of D-(-) tartaric acid (1 eq) in methanol (10 mL) was added with stirring at room temperature. The reaction mixture was further stirred overnight at room temperature. Adding dry ethyl ether precipitated the tartrate salts (**19**,**22**,**24**,**26**).

*3.1.10.1. 2-(2-methyl-5-nitro-1H-imidazol-1-yl)ethyl pyrrolidine-1-carbodithioate (**19**).* Brown solid; Yield 86%; Mp 138–139 °C, IR (KBr): 3020, 1529, 1436, 1216, 760, cm^{-1}; ESI-MS: *m/z* 300 (M^+), 301 ($M^+ + 1$); ^1H NMR (300 MHz, $CDCl_3$, δ ppm): δ 7.95 (s, 1H), 4.67 (t, 2H, *j* = 6.9 Hz), 3.93 (t, 2H, *j* = 6.9 Hz), 3.70–3.62 (m, 4H), 2.59 (s, 3H), 2.15–1.96 (m, 4H); ^{13}C NMR (75 MHz, $CDCl_3$, δ ppm): 190.8, 151.4, 138.5, 133.4, 55.5, 50.9, 45.3, 34.9, 26.2, 24.4, 14.9; Anal. Calcd. for $C_{11}H_{16}N_4O_2S_2$ (300): C, 43.94; H, 5.32; N, 18.64; Found: C, 43.69; H, 5.54; N, 18.46; Tartrate salt mp 92–93 °C.

*3.1.10.2. 2-(2-methyl-5-nitro-1H-imidazol-1-yl)ethyl morpholine-4-carbodithioate (**20**).* Off white solid; Yield 76%; Mp 127–128 °C; IR (KBr): 3018, 1530, 1264, 762 cm^{-1}; ESI-MS: *m/z* 316 (M^+), 317 ($M^+ + 1$); ^1H NMR (300 MHz, $CDCl_3$, δ ppm): δ 7.94 (s, 1H), 4.60 (t, 2H, *j* = 6.9 Hz), 4.43–4.23 (m, 2H), 4.01–3.89 (m, 2H), 3.75–3.68 (m, 6H), 2.57 (s, 3H); Anal. Calcd. for $C_{11}H_{16}N_4O_3S_2$ (316): C, 41.76; H, 5.10; N, 17.71; Found: C, 41.68; H, 5.33, N, 17.63.

*3.1.10.3. 2-(2-methyl-5-nitro-1H-imidazol-1-yl)ethyl piperidine-1-carbodithioate (**21**).* Light yellow solid; Yield 76%; Mp 109–110 °C; IR (KBr): 3020, 1529, 1363, 1216, 761, cm^{-1}; ESI-MS: *m/z* 314 (M^+), 315 ($M^+ + 1$); ^1H NMR (300 MHz, $CDCl_3$, δ ppm): δ 7.96 (s, 1H), 4.62 (t, 2H, *j* = 6.8 Hz), 4.33–4.25 (m, 2H), 3.91–3.87 (m, 2H), 3.71 (t, 2H, *j* = 6.9 Hz), 2.58 (s, 3H), 1.82–1.60 (m, 6H); Anal. Calcd. for $C_{12}H_{18}N_4O_2S_2$ (314): C, 45.84; H, 5.77; N, 17.82; Found: C, 46.17; H, 6.04; N, 17.42.

*3.1.10.4. 2-(2-methyl-5-nitro-1H-imidazol-1-yl)ethyl dimethylcarbamodithioate (**22**).* Yellow solid; Yield 79%; Mp 109–110 °C; IR (KBr):

3020, 1530, 1261, 786, cm^{-1}; ESI-MS: *m/z* 274 (M^+), 275 ($M^+ + 1$); ^1H NMR (300 MHz, $CDCl_3$, δ ppm): δ 7.93 (s, 1H), 4.58 (t, 2H, $j = 7.0$ Hz), 3.66 (t, 2H, $j = 6.9$ Hz), 3.55 (s, 3H), 3.36 (s, 3H), 2.56 (s, 3H); ^{13}C NMR (50 MHz, $CDCl_3$, δ ppm): δ 195.3, 151.5, 138.6, 133.5, 45.8, 45.2, 41.8, 36.0, 14.9; Anal. Calcd. for $C_9H_{14}N_4O_2S_2$ (274): C, 39.40; H, 5.14; N, 20.42; Found: C, 39.66; H, 5.36; N, 20.66; Tartrate salt mp 88–89 °C.

*3.1.10.5. 2-(2-methyl-5-nitro-1H-imidazol-1-yl)ethyl diethylcarbamodithioate (**23**).* Light yellow solid; Yield 70%; Mp 65–66 °C; IR (KBr): 2926, 1526, 1267, 739 cm^{-1}; ESI-MS: *m/z* 303 ($M^+ + 1$); ^1H NMR ($CDCl_3$, 300 MHz, δ ppm): δ 7.99 (s, 1H), 4.63 (t, 2H, $j = 6.9$ Hz), 4.07–4.00 (m, 2H), 3.76–3.74 (m, 2H), 3.68 (t, 2H, $j = 6.9$ Hz), 2.57 (s, 3H), 1.28 (t, 6H, $j = 7.9$ Hz); ^{13}C NMR (75 MHz, $CDCl_3$, δ ppm): 193.7, 151.4, 138.5, 133.4, 50.15, 47.18, 45.2, 35.6, 14.9, 12.8, 11.8; Anal. Calcd. for $C_{11}H_{18}N_4O_2S_2$ (302): C, 43.69; H, 6.00; N, 18.53; Found: C, 43.71; H, 6.22, 18.66.

*3.1.10.6. 2-(2-methyl-5-nitro-1H-imidazol-1-yl)ethyl 4-(pyridin-2-yl)piperazine-1-carbodithioate (**24**).* Yellow solid; Yield 65%; Mp 144–145 °C; IR (KBr): 3021, 1597, 1522, 1216, 764 cm^{-1}; ESI-MS: *m/z* 393 ($M^+ + 1$); ^1H NMR (300 MHz, $CDCl_3$, δ ppm): δ 8.18–8.16 (m, 1H), 7.93 (s, 1H), 7.54–7.48 (m, 1H), 6.69–6.60 (m, 2H), 4.59 (t, 2H, $j = 6.9$ Hz), 4.54–4.32 (m, 2H), 4.13–3.93 (m, 2H), 3.73–3.68 (m, 6H), 2.55 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$, δ ppm): δ 194.8, 158.0, 151.0, 147.6, 138.2, 137.6, 133.1, 113.9, 106.8, 44.7, 44.1, 35.0, 14.5; Anal. Calcd. for $C_{16}H_{20}N_6O_2S_2$ (392): C, 48.96; H, 5.14; N, 21.41; Found; C, 48.77; H, 5.38; N, 21.19; Tartrate salt hygroscopic.

*3.1.10.7. 2-(2-methyl-5-nitro-1H-imidazol-1-yl)ethyl 4-(pyrimidin-2-yl) piperazine-1-carbodithioate (**25**).* Yellow solid; Yield 78%; Mp 179–180 °C; IR (KBr): 3020, 1586, 1529, 1261, 761 cm^{-1}; ESI-MS: *m/z* 394 ($M^+ + 1$); ^1H NMR (300 MHz, $CDCl_3$, δ ppm): δ 8.33–8.31 (m, 2H), 7.93 (s, 1H), 6.58–6.54 (m, 1H), 4.60 (t, 2H, $j = 6.9$ Hz), 4.50–4.31 (m, 2H), 4.19–3.94 (m, 6H), 3.72 (t, 2H, $j = 6.9$ Hz), 2.56 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$, δ ppm): δ 195.3, 161.3, 158.0, 151.4, 138.6, 133.5, 111.0, 45.2, 43.1, 35.4, 14.9; Anal. Calcd. for $C_{15}H_{19}N_7O_2S_2$ (393): C, 45.79; H, 4.87; N, 24.92; Found: C, 45.64; H, 4.84; N, 25.13.

*3.1.10.8. 2-(2-methyl-5-nitro-1H-imidazol-1-yl)ethyl 4-(2-methoxyphenyl)piperazine-1-carbodithioate (**26**).* Yellow solid; Yield 70%; Mp 131–132 °C; IR (KBr): 3020, 1594, 1530, 1216, 760 cm^{-1}; ESI-MS: *m/z* 422 ($M^+ + 1$); ^1H NMR (200 MHz, $CDCl_3$, δ ppm): δ 7.93 (s, 1H), 7.09–7.00 (m, 1H), 6.93–6.85 (m, 3H), 4.60 (t, 2H, $j = 6.9$ Hz), 4.53–4.42 (m, 2H), 4.19–3.95 (m, 2H), 3.86 (s, 3H), 3.70 (t, 2H, $j = 6.9$ Hz), 325–3.04 (m, 4H), 2.56 (s, 3H); Anal. Calcd. for $C_{18}H_{23}N_5O_3S_2$ (421): C, 51.29; H, 5.50; N, 16.61; Found: C, 51.50; H, 5.33; N, 16.49; Tartrate salt mp 99–100 °C.

*3.1.10.9. 2-(2-methyl-5-nitro-1H-imidazol-1-yl)ethyl 4-(4-nitrophenyl)piperazine-1-carbodithioate (**27**).* Orange solid; Yield 75%; Mp 199–200 °C; IR (KBr): 3114, 2919, 1595, 1318, 1254, 745 cm^{-1}; ESI-MS: *m/z* 437 ($M^+ + 1$); ^1H NMR (300 MHz, TFA-d, δ ppm): δ 8.04 (d, 2H, $j = 8.6$ Hz), 7.82 (s, 1H), 7.48 (d, 2H, $j = 8.7$ Hz), 4.54 (t, 2H, $j = 6.2$ Hz), 4.40–4.21 (m, 4H), 3.55–3.48 (m, 6H), 2.52 (s, 3H); Anal. Calcd. for $C_{17}H_{20}N_6O_4S_2$ (436): C, 46.78; H, 4.62; N, 19.25; Found: C, 46.61; H, 4.75; N, 19.06.

*3.1.10.10. 2-(2-methyl-5-nitro-1H-imidazol-1-yl)ethyl 4-methylpiperazine-1-carbodithioate (**28**).* Brown solid; Yield 65%; Mp 105–106 °C; IR (KBr): 3020, 1531, 1217, 761 cm^{-1}; ESI-MS: *m/z* 330 ($M^+ + 1$); ^1H NMR (300 MHz, $CDCl_3$, δ ppm): δ 7.89 (s, 1H), 4.61–4.49 (m, 2H), 4.45–4.24 (m, 2H), 4.04–3.82 (m, 2H), 3.64 (t, 2H, 6.9 Hz), 2.53–246 (m, 7H), 2.34 (s, 3H). Anal. Calcd. for $C_{12}H_{19}N_5O_2S_2$ (328): C, 43.75; H, 5.81; N, 21.26; Found: C, 43.66; H, 5.65; N, 21.32.

3.2. Biology

3.2.1. Spermicidal activity

Spermicidal test was adapted from the standard procedure [30]. Briefly, the test compounds were dissolved in a minimum volume of DMSO and diluted with physiological saline (0.85% NaCl in distilled water) to make a 1.0% solution. A spermicidal test was performed with each compound solution and for this purpose 0.05 mL of liquefied human semen was added to 0.25 mL of test solution and vortexed for 10 s at low speed. A drop of the mixture was then placed on a microscope slide, covered with a cover glass and examined under a phase contrast microscope in five fields of vision. The percentage of motile spermatozoa was determined by visual scoring in the next 30–40 s and recorded (Table 1). N-9 was used as reference control.

3.2.2. Anti-Trichomonas activity

T. vaginalis parasites to be used in drug susceptibility assays were grown in TYM medium [31] supplemented with 10% Fetal Calf Serum (FCS), vitamin mixture and 100 U/mL penicillin/streptomycin mixtures, at 37 °C in 15 mL tubes for one day following regular subculturing, and were in the log phase of growth. The cultures routinely attained a concentration of 2×10^7 cells/mL in 48 h. An inoculum of 1×10^4 cells per tube was used for maintenance of the culture. *In vitro* drug susceptibility assays were carried out using the standard procedure [32]. Stock solutions (100 μg/μL) of test compounds were prepared in DMSO. These stock solutions were serially diluted with TYM medium to obtain concentrations up to 0.1 μg/mL in 48-well plates. DMSO/TYM was used as vehicle in control wells. Parasites (5×10^4 trophozoites/L) were added to these wells and incubated anaerobically at 37 °C. Cells were checked for viability at different time intervals from 3 to 48 h under the microscope at 200× magnification. Viability of the cells was determined by trypan blue exclusion assay. Minimum concentration of the test agent at which all cells were found dead in 48 h was considered as its minimum inhibitory concentration (MIC). Metronidazole (Sigma-Aldrich), the anti-*trichomonas* drug presently available in the market, was used as reference standard. The experiment was repeated three times to confirm the MIC (Table 1).

3.2.3. Antifungal activity

The MIC of compounds were determined [33] by broth microdilution technique as per guide lines of National Committee for Clinical Laboratory Standards using RPMI 1640 media buffered with MOPS [3-(N-Morpholino)propanesulfonic acid]. Starting inoculums of test culture was $1–5 \times 10^3$ CFU/mL. Micro titer plates were incubated at 35 °C. MICs were recorded after 48 h of incubation (Table 2).

3.2.4. Cyto-toxicity assay using human cervical (HeLa) cell line

The cyto-toxicity of compounds were assessed [12] against human cervical (*HeLa*) cell line using the MTT (3-[4,5-dimethyl thiazol-2-yl]-2,5-diphenyltetrazolium bromide) based colorimetric assay. *HeLa* cells (procured from National Centre for Cell Science, Pune, India) seeded at a density of 5.0×10^4 per well in 96 well plates were incubated in culture medium (DMEM with 10% FCS) for 24 h at 37 °C in 5%CO_2/95% air atmosphere. After 24 h, culture medium was replaced with fresh medium containing dilutions of test compounds (0–200 μg/mL). Vehicle (10% DMSO) was added to 0 μg/mL (control) wells. After incubation for another 24 h, 10 μL of MTT solution (5 mg/mL in PBS, pH 7.4) was added to each well. The formazan crystals formed inside the viable cells were solubilized in DMSO and the OD was recorded at 540 nm in a microplate reader (Microquant, Bio-Tek, USA). Cyto-toxicity at the highest concentration tested (200 μg/mL) has been displayed in Fig. 2.

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Effects of metronidazole analogues on *Giardia lamblia*: experimental infection and cell organization ☆

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ABSTRACT

The chemotherapeutic agents used for the treatment of giardiasis are often associated with adverse side effects and are refractory cases, due to the development of resistant parasites. Therefore the search for new drugs is required. We have previously reported the giardicidal effects of metronidazole (MTZ) and its analogues (MTZ-Ms, MTZ-Br, MTZ-N_3, and MTZ-I) on the trophozoites of *Giardia lamblia*. Now we evaluated the activity of some giardicidal MTZ analogues in experimental infections in gerbils and its effects on the morphology and ultrastructural organization of *Giardia*. The giardicidal activity in experimental infections showed ED_{50} values significantly lower for MTZ-I and MTZ-Br when compared to MTZ. Transmission electron microscopy was employed to approach the mechanism(s) of action of MTZ analogues upon the protozoan. MTZ analogues were more active than MTZ in changing significantly the morphology and ultrastructure of the parasite. The analogues affected parasite cell vesicle trafficking, autophagy, and triggered differentiation into cysts. These results coupled with the excellent giardicidal activity and lower toxicity demonstrate that these nitroimidazole derivates may be important therapeutic alternatives for combating giardiasis. In addition, our results suggest a therapeutic advantage in obtaining synthetic metronidazole analogues for screening of activities against other infectious agents.

1. Introduction

Giardia lamblia is a parasitic protozoan that colonizes the human intestinal tract causing a wide clinical spectrum disorder called giardiasis. The disease is a zoonosis which is considered an important public health problem in many countries worldwide. It infects about 200 million people in Asia, Africa, and Latin America (Yason and Rivera, 2007). *G. lamblia* is a major cause of diarrhea among children and travelers (Buret, 2008).

Infected individuals may be asymptomatic or present dehydration-causing diarrhea and abdominal discomfort. Giardiasis can produce chronic diarrhea, lasting for several months, which may result in malabsorption and weight loss, contributing to the increased mortality of individuals who are malnourished or immune deficient in the first 3 years of life (Buret et al., 2002; Ankarklev et al., 2010; Wensaas et al., 2010; Cotton et al., 2011). Both host and parasite factors contribute to the pathogenesis of giardiasis. Malabsorption, maldigestion, chloride hypersecretion, and increased rates of small intestinal transit are the main factors involved in the onset of diarrhea (Buret, 2008; Cotton et al., 2011).

Therefore *G. lamblia* has been implicated in the disturbance of physical (Farthing et al., 1986; Simsek et al., 2004) and cognitive (Thompson et al., 1993) development among children. It is estimated that the incidence of giardiasis in the world reached 1 billion cases, constituting one of the most common protozoan infection (Wright et al., 2003). Nevertheless, it is a neglected disease. A variety of chemotherapeutic agents have been used in the treatment of giardiasis. However, most of the drugs used display significant side effects and are contraindicated in some cases. Moreover, *Giardia* is able to develop resistance to these agents (Wright et al., 2003; Müller et al., 2000). Giardiasis was included in the 'Neglected Diseases Initiative' (Savioli et al., 2006) highlighting the need for new effective nontoxic giardicidal drugs.

The introduction of nitroheterocyclic drugs, in the 1950s, represented a new era in the treatment of bacterial and protozoan infections. Metronidazole (1- β-hydroxyethyl-2-methyl-5-nitroimidazole) is currently the most widely used drug for the treatment of infections caused by *G. lamblia*, *Entamoeba histolytica*, *Trichomonas vaginalis*, and *Blastocystis* spp. (Upcroft and Upcroft, 2001; Busatti et al., 2009; Leitsch

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et al., 2011; Mirza et al., 2011). Cases of recurrent symptoms and resistance have been documented in all these parasites (Upcroft et al., 2006; Bansal et al., 2006; Tejman-Yarden et al., 2011), encouraging the advancement of research on alternative drugs against these parasites.

Metronidazole (MTZ) analogues have been developed and tested against different microorganisms, and some of them have proved effective against *Giardia*, *Trichomonas*, and *Entamoeba histolytica* (Upcroft et al., 2006; Barbosa et al., 2006; Busatti et al., 2007).

Previously, we reported the activity of MTZ analogues against *G. lamblia* (Busatti et al., 2007). Here we evaluated the giardicidal activity of some MTZ analogues in experimentally infected gerbils and employed an ultrastructural analysis as an instrumental tool to clarify the mechanisms of action of these potential drugs upon the parasite.

2. Material and methods

2.1. Synthesis of MTZ analogues

MTZ analogues were obtained by reactions as described previously (Busatti et al., 2007). The purity of these compounds was evaluated by thin layer chromatography on silica gel plates. The structures were confirmed by spectral data analysis (RMN^{1}H and RMN^{13}C). The dose–response curves were obtained by associating axenic cultures of *Giardia* with increasing concentrations of MTZ, MTZMs, MTZN$_3$, MTZ-Br, and MTZI, ranging from 0.08 to 30 μmol/L.

2.2. Cultures and growth conditions

G. lamblia Portland strain (ATCC 30888) was used in all experiments. It was kept axenically at 37 °C in Diamond's modified TYI-S-33 medium (Keister, 1983) supplemented with heat-inactivated bovine serum at 10%. To quantify the drug's action, 1.5×10^5 trophozoites of *Giardia* were grown in culture plates of 24 wells (Nunc, Berkeley, CA, USA) in CO_2 atmosphere at 37 °C for 48 h.

2.3. Antigiardial activity in vivo

Giardicidal activity, in vivo, of MTZ analogues was assessed by determining the parasitic load of trophozoites as previously described (Belosevic et al., 1983; Araújo et al., 2008) with some modifications. The activity of MTZ analogues on *Giardia* trophozoites was evaluated in vivo using gerbils (*Meriones unguiculatus*) as an experimental model. The experiments were performed in compliance with the guidelines of the Institutional Animal Care and Committee on Ethics of Animal Experimentation (Comitê de Ética em Experimentação Animal–CETEA, national guidelines Lei 11.794, de 8 de outubro de 2008) of Universidade Federal de Minas Gerais (UFMG; protocol number 181/2008, approved on 03/04/2009). Animals aged 4–8 weeks of both sexes were used. They were divided into groups of 6 animals for each compound: a negative control group (in the absence of nitroimidazoles), a group that received the vehicle (phosphate buffered saline [PBS, pH 7.2], containing dimethyl sulfoxide [DMSO, 0.05%]); a positive control group (in the presence of metronidazole); and 5 test groups, where animals infected with *G. lamblia* were treated with 0.1 to 6.0 μmol/kg of MTZ and its analogues, MTZ-I and MTZ-Br.

For the inhibition assay, 1×10^6 *G. lamblia* trophozoites in 1 mL of PBS were inoculated in gerbils by gavage. Six days after inoculation, the animals were treated intragastrically with 1 mL of the nitroimidazoles, dissolved in PBS containing 0.05% DMSO. Two days after the treatment with drugs (8 days after inoculation), the animals were sacrificed and 18 cm of the small intestine of each animal was removed, opened longitudinally, and placed in glass tubes containing 10 mL of cold PBS for 20 min. ED_{50}, which is the dose leading to 50% parasite growth inhibition, compared to growth in the control, was determined for each compound.

2.4. Transmission electron microscopy

Parasites were fixed in 4% paraformaldehyde (Polysciences, Warrington, PA, USA), 2.5% glutaraldehyde (Polysciences), 4% sucrose in 0.1 mol/L sodium cacodylate buffer (pH 7.2) for at least 1 h, post-fixed in 1% osmium tetroxide (Polysciences) and 0.8% potassium ferricyanide in the same buffer for 40 min, dehydrated in acetone series, and embedded in Polybed resin (Polysciences). Thin sections were stained with 2% uranyl acetate for 20 min and with 1% lead citrate for 5 min and observed under a Zeiss 900 transmission electron microscope (Carl-Zeiss, Oberkochen, Germany).

The morphometric analysis of peripheral vesicles of trophozoites was performed before and after MTZ-I treatments. The area determination was made based on the limits of these organelles, using the software SIS iTEM (SIS iTEM, Palatka, FL, USA). Three vesicles per cell were selected randomly on at least 20 cells observed on ultrathin sections. The data plotted in GraphPad Prism 5.0 (GraphPad, San Diego, CA, USA) are represented as the mean ± SEM and were analyzed by Student's *t* test with a significance level of $P < 0.05$.

2.5. Statistical analysis

Each experiment was done at least 3 times in triplicate. Analysis of variance (ANOVA) was used to analyze differences between IC_{50} (dose required for 50% growth inhibition in vitro) and ED_{50} (dose required to inhibit 50% of organisms growth in vivo) values. *P* values below 0.05 were considered statistically significant.

3. Results

3.1. Antigiardial activity

The IC_{50} of MTZ-Ms (0.69 ± 0.05), MTZ-N3 (0.70 ± 0.16), MTZ-I (0.40 ± 0.03), and MTZ-Br (0.28 ± 0.04) tested presented higher giardicidal activity when compared with MTZ (1.96 ± 0.13). MTZ-Br and MTZ-I were the most active ($P < 0.001$), so they were chosen to determine the antigiardial activity in vivo.

Both the MTZ analogues MTZ-I and MTZ-Br were able to significantly ($P < 0.001$) reduce the *G. lamblia* parasite load in infected gerbils. MTZ-Br and MTZ-I had greater giardicidal activity, with ED_{50} values significantly lower than the MTZ (Table 1).

3.2. Transmission electron microscopy

Ultrastructural analysis of untreated control *G. lamblia* trophozoite fixed after 48 h of incubation in the presence of 0.05% DMSO did not induce alterations in the ultrastructure of the protozoan (Fig. 1A), indicating that this solvent concentration was nontoxic.

Trophozoites incubated with MTZ showed evident ultrastructural disorganization (Fig. 1B) characterized by the centripetal displacement of the peripheral vesicles and internalization of the cytoskeletal components of the flagella and adhesive disk into the cytoplasmic matrix.

Table 1
Activity in vivo of MTZ analogues against *G. lamblia* in gerbils.[a]

Compound	ED_{50} (μmol/kg)[b]
MTZ[c]	0.74 (0.78–0.72)
MTZ-Br	0.51 (0.53–0.49)
MTZ-I	0.38 (0.42–0.34)

[a] Results are expressed as mean ($n = 6$).
[b] Dose required to inhibit 50% of organism growth with 95% confidence limits.
[c] Positive control.

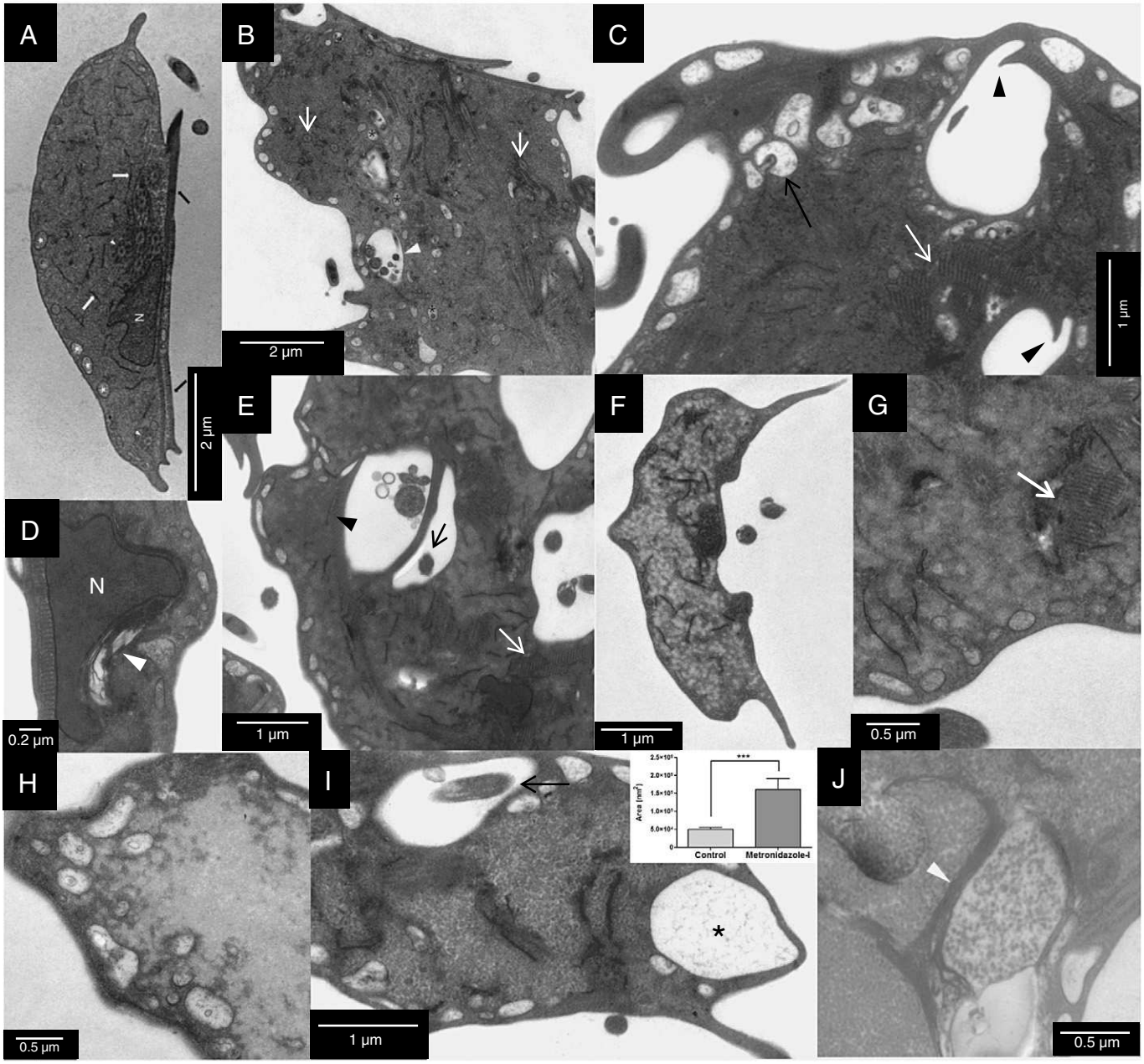

Fig. 1. (A) Cross section of *Giardia lamblia* trophozoite incubated with 0.05% DMSO showing characteristic ultrastructure, with the presence of peripheral vesicles (*), endoplasmic reticulum cisternae (white arrows), nucleus (N), adhesive disk (black arrows), and axonemes of the flagella (arrowheads) within the homogeneously electron-dense cytoplasm. (B) *G. lamblia* trophozoites incubated with 0.85 µmol/L of metronidazole for 48 h, with ultrastructural disorganization, centripetal displacement of peripheral vesicles (*), and flagellar internalization (arrowhead) as well as numerous cytoplasmic axonemes (arrows). (C) Trophozoites incubated for 48 h in the presence of 0.51 µmol/L of metronidazole-N3 displaying peripheral vesicle dilatation and interdigitation (black arrow), as well as concave regions of adhesion disk engulfment areas (arrowheads) and disassembly of its cytoskeleton components (white arrow). (D) Trophozoites incubated for 48 h in the presence of 0.51 µmol/L of metronidazole-N3 displaying the formation of extensive concentric arrays of endoplasmic reticulum cisternae (arrowhead) circumscribing the nuclei (N). (E) Cells incubated for 48 h in the presence of 0.48 mmol/L of metronidazole-MS showed marked disorganization of the cellular architecture of trophozoites and internalization of the flagella (black arrow), adhesive disk (arrowhead), and its components' disassembly (white arrow). (F) We also observed a lower cytoplasmic electrondensity and the absence of glycogen granules. (G) and (H) *G. lamblia* trophozoites treated with 0.32 µmol/L of metronidazole-Br for 48 h. We observed internalization and disaggregation of adhesive disk cytoskeleton components (G, arrow), heterogeneous cytoplasmic granulosity, and electrondensity properties as many parasites displayed remarkable cytoplasmic extraction (H), resulting in altered cell architecture. (I) and (J) *G. lamblia* trophozoites treated with 0.26 µmol/L of metronidazole-I displayed remarkably swollen vesicles (I, *), which may be involved in the internalization of the flagella (arrow). Morphometry of the peripheral vesicles revealed that the average area in the 0.26 µmol/L metronidazole-I–treated images was significantly (***, $P < 0.001$) enhanced (I, inset). Several cells presented autophagic process (J), characterized by concentric membranes (arrowhead) surrounding the cytoplasmic material presumably in degradation.

Parasites incubated with MTZ-N_3 displayed large areas of adhesive disk internalization and peripheral vesicle interdigitation (Fig. 1C). Several cells presented multilayered membranes stacked in the perinuclear area (Fig. 1D).

Altered parasite cell architecture and adhesive disk internalization were also found in trophozoites incubated with MTZ-Ms (Fig. 1E). Lower cytoplasmic electrodensity and absence of glycogen granules were also observed (Fig. 1F). Heterogeneous cytoplasmic granulosity and electrondensity properties were observed in *Giardia* trophozoites treated with 0.32 µmol/L metronidazole-Br for 48 h (Fig. 1G), as well as internalization of adhesive disk cytoskeleton components (arrowhead). Many parasites displayed remarkable cytoplasmic extraction (Fig. 1H), resulting in altered cell architecture.

Parasites grown in the presence of MTZ-I presented remarkably large vesicles, which may be involved in the internalization of the flagella (Fig. 1I). Morphometric analysis of 0.26 μmol/L of MTZ-I–treated parasites revealed 3-fold ($P < 0.001$) enlargement of sectioned peripheral vesicle areas as compared to controls (Fig. I, insert). Autophagic vacuole formation surrounding the cytoplasmic material presumably in degradation was also observed (Fig. 1J).

4. Discussion

Despite its significant morbidity causing considerable socioeconomic impact, especially in underdeveloped countries, giardiasis is largely overlooked.

Besides sanitation, disease control could be accomplished by vaccination (Olson et al., 2000) and with new drugs to avoid the resistance phenomenon (Argüello-García et al., 2009; Leitsch et al., 2011). MTZ analogues are efficient giardicidal compounds and could become an alternative for the treatment of giardiasis (Upcroft et al., 2006; Busatti et al., 2007). Thus, we evaluated the giardicidal activity of some MTZ analogues in experimentally infected gerbils and its effects on the morphology and ultrastructural organization of *Giardia*.

All analogues tested displayed higher anti-*Giardia* activity than the MTZ. The analogues were able to significantly reduce the parasite load of gerbils infected with *G. lamblia*. In an electrochemical study, these analogues showed greater reduction potential for MTZ-Br followed by MTZ-I and MTZ (Cavalcanti et al., 2004). These results highlight the reduction potential as a chemical giardicidal property relevant to the activity of these compounds. Reduction leads to the formation of toxic nitro radicals, which are responsible for the destabilization of DNA and the production of other toxic radicals that react with essential cellular components, interfering with cellular metabolism (Edwards, 1993; Leitsch et al., 2011).

The transmission electron microscopy revealed that trophozoites of *G. lamblia* treated with MTZ and its analogues showed a marked change in cellular architecture with evident ultrastructural disorganization. Trophozoites treated with the analogues showed changes similar to those observed in parasites treated with MTZ, such as centripetal displacement of the peripheral vesicles, internalization of the flagella and cytoskeletal components of the adhesive disk, vacuolization, and dilation and extraction of peripheral cytoplasmic vesicles, indicating that the mechanisms of action of these compounds caused similar metabolic changes in the parasite.

Antiparasitic drugs may affect the protozoa endocytic (Bernardes et al., 2000) and/or autophagic pathways (Vannier-Santos et al., 1999; Vannier-Santos and de Castro, 2009). The formation of concentric membranes and myelin figures is indicative of autophagic processes; these changes were particularly observed in trophozoites treated with MTZ-I analogues. The engulfment of digit-form processes between adjacent peripheral vesicles might characterize microautophagy triggering as vacuolar tubular invaginations may be involved in autophagy (Müller et al., 2007). Interestingly, the *Giardia* lysosomal functions may be performed by endoplasmic reticulum–like tubulovesicular compartments (Abodeely et al., 2009). The formation of such concentric cisternae in the perinuclear area may be related to the endoplasmic reticulum nature of the nuclear envelop. In this regard, it is noteworthy that herpes simplex virus 1–induced autophagy may be associated with the formation of concentric cisternae from the macrophage nuclear envelope (English et al., 2009). The internalization of the flagella and adhesion disk components is presumably associated with parasite encystment due to the drug-induced stress (Maia et al., 2008).

In previous studies, we observed that the analogues of MTZ were not cytotoxic in vitro to human cells in a concentration range from 0.2 to 6.4 μmol/L (Busatti et al., 2007). These results coupled with the excellent giardicidal activity in vivo and in vitro demonstrate that these nitroimidazole derivates may be important therapeutic alternatives to combat giardiasis. Furthermore, the present results suggest a therapeutic advantage with obtaining synthetic analogues of MTZ for screening of activities against other infectious agents.

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