

NEW DERIVATIVES OF 5-NITROIMIDAZOLE:
SYNTHESIS AND ANTIPARASITIC ACTIVITY

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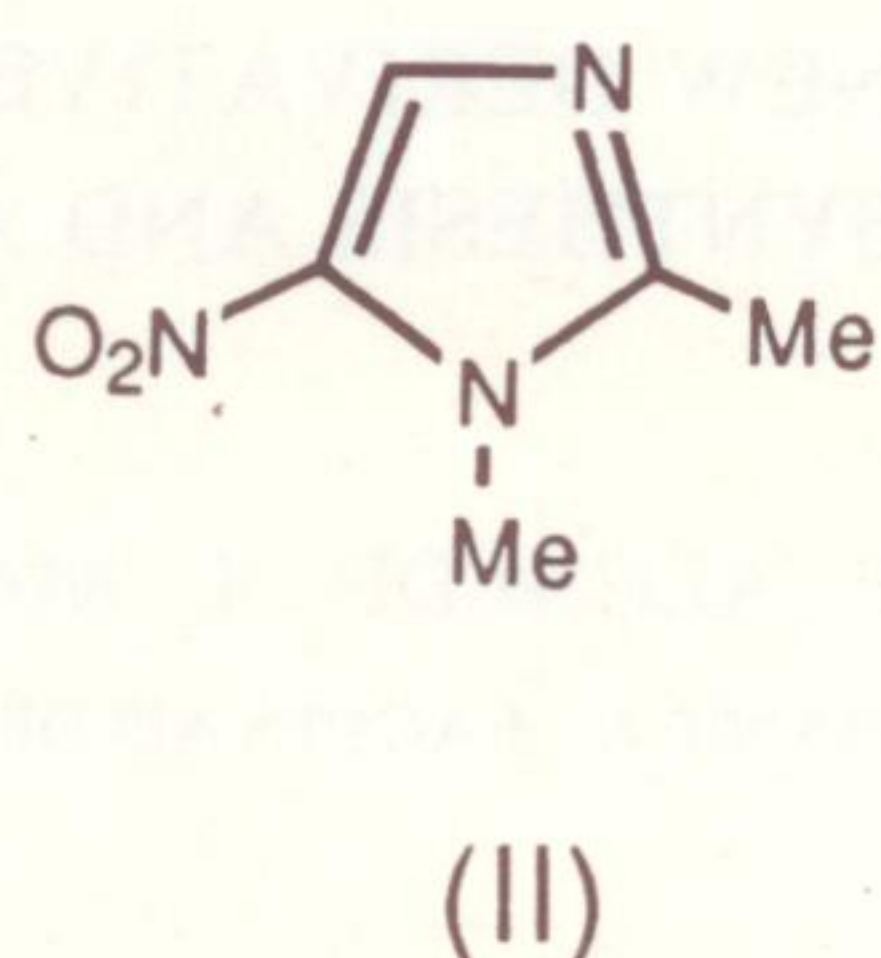
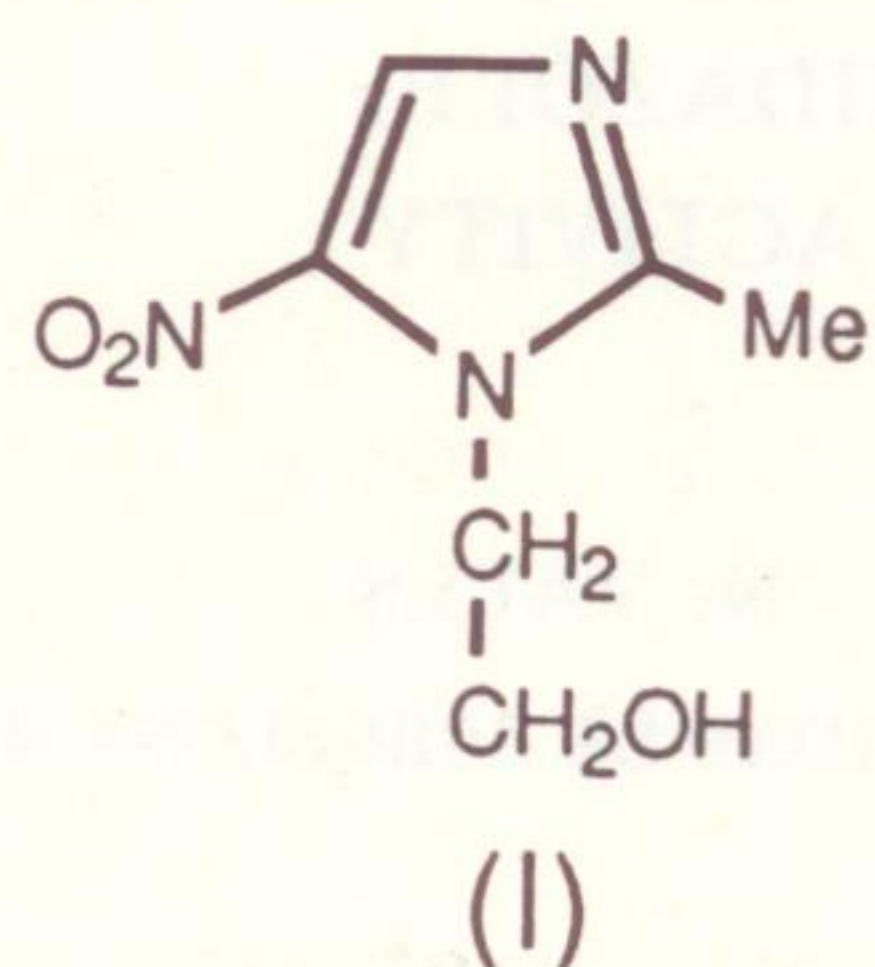
SUMMARY — *The synthesis and the antiparasitic evaluation of twelve new 5-nitroimidazole derivatives has been carried out. The most effective compounds were the less hydrophilic pyridinium and imidazolium salts (IV), (V) and (X), and above all the tetrahydropyridine derivatives (XII) and (XIII).*

RIASSUNTO — *Vengono riportate le sintesi di dodici nuovi derivati 5-nitroimidazolici e la valutazione della loro attività antiparassitaria. I composti che si sono dimostrati più attivi sono i sali di piridinio ed imidazolio (IV), (V) e (X), meno idrofili e, ancora di più, i derivati tetraidropiridinici (XII) e (XIII).*

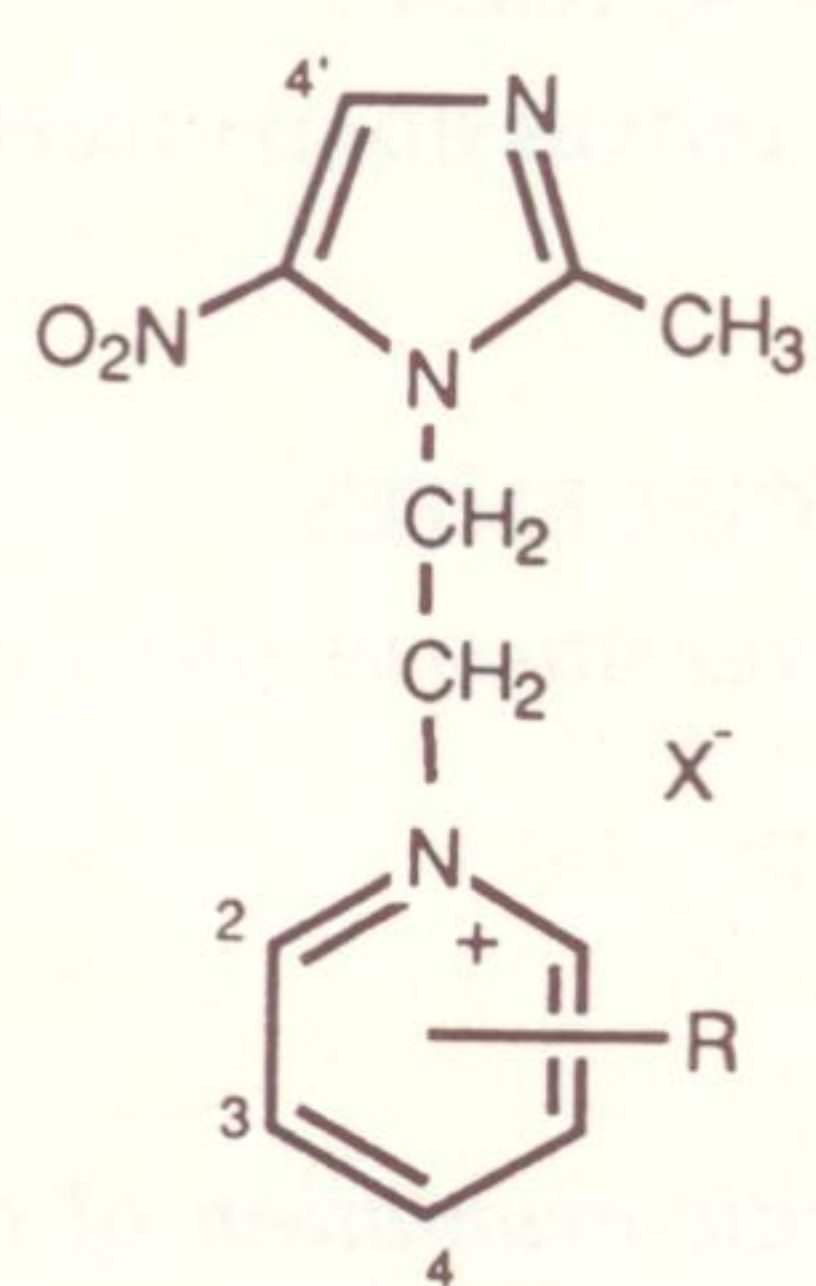
Introduction

5-Nitroimidazole derivatives have a wide range of biological properties that provide an increasing number of therapeutic applications. The major clinical use of these compounds is for diseases caused by bacteria and protozoa, and also for radio-sensitization of hypoxic cells in cancer chemotherapy (1,2). The purpose of the present study is to ascertain the effect of highly polar groups (pyridinium and imidazolium) on the biological

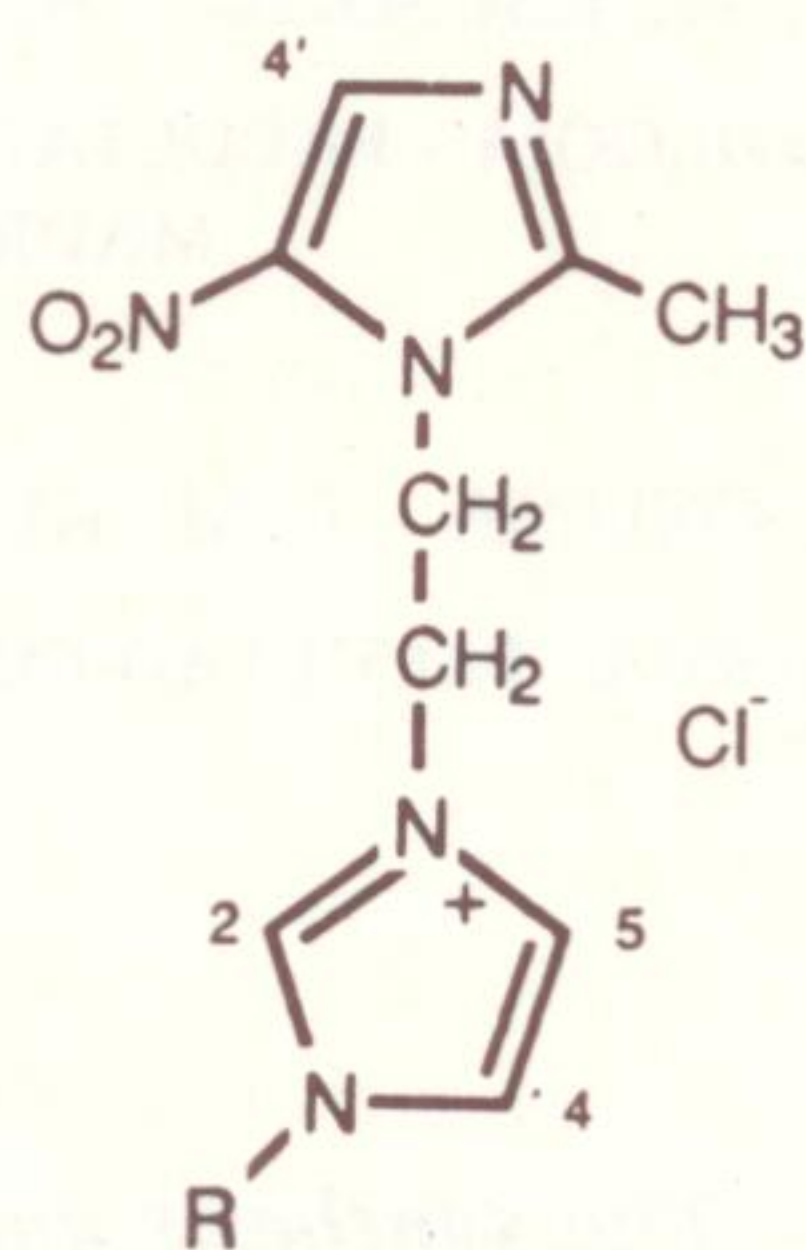
properties of 5-nitroimidazoles and, comparatively to study the effect of the less polar tetrahydropyridines. All the molecules reported here are structurally related to metronidazole (I) and dimetridazole (II) (1) and their potential activity was evaluated against eight different parasites.



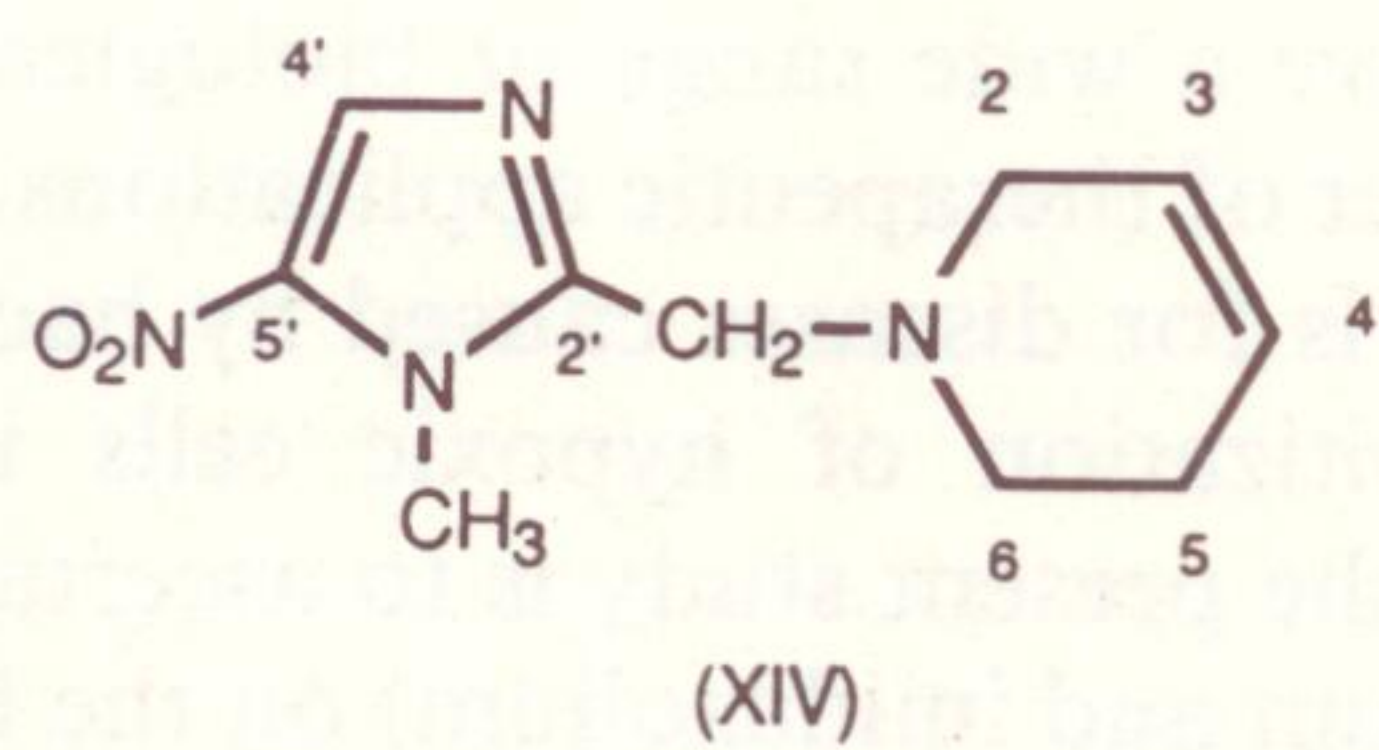
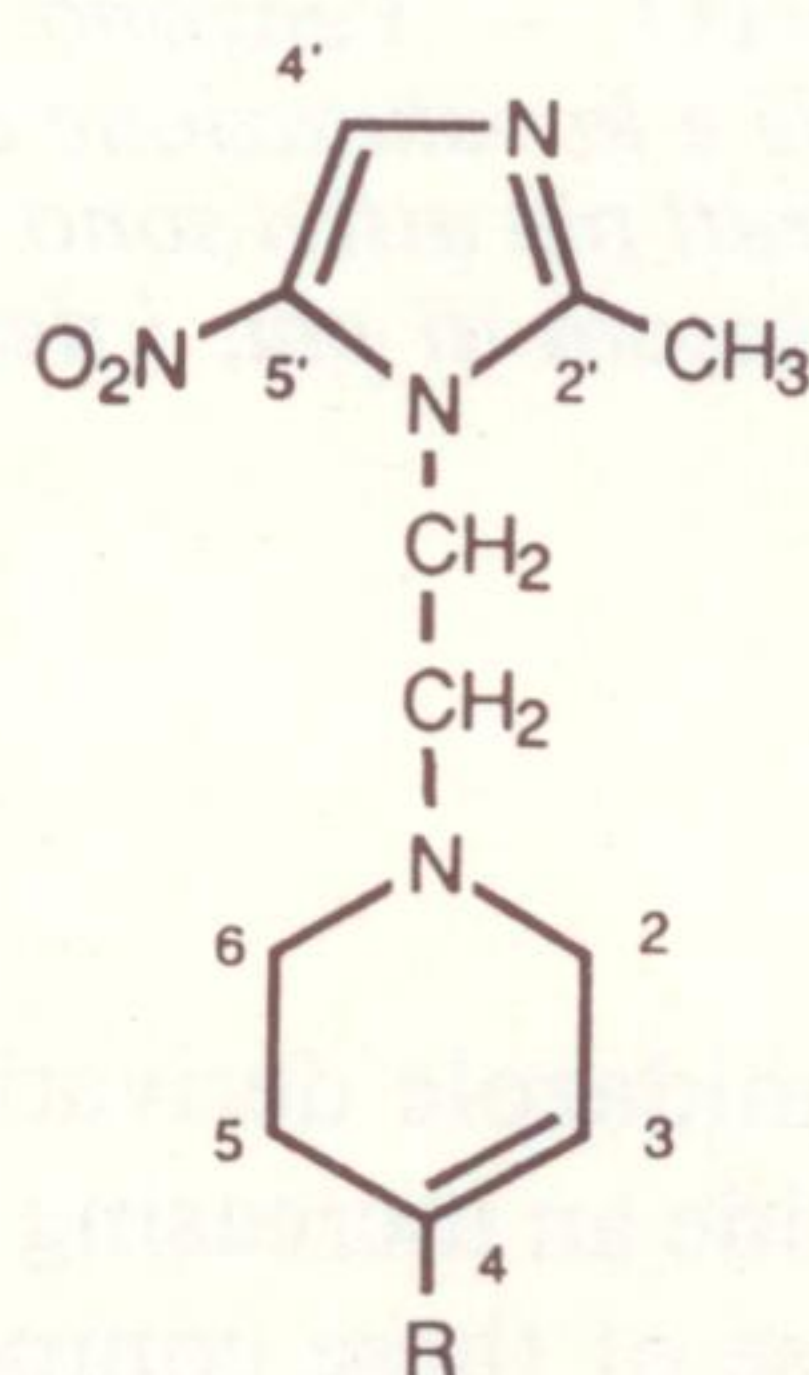
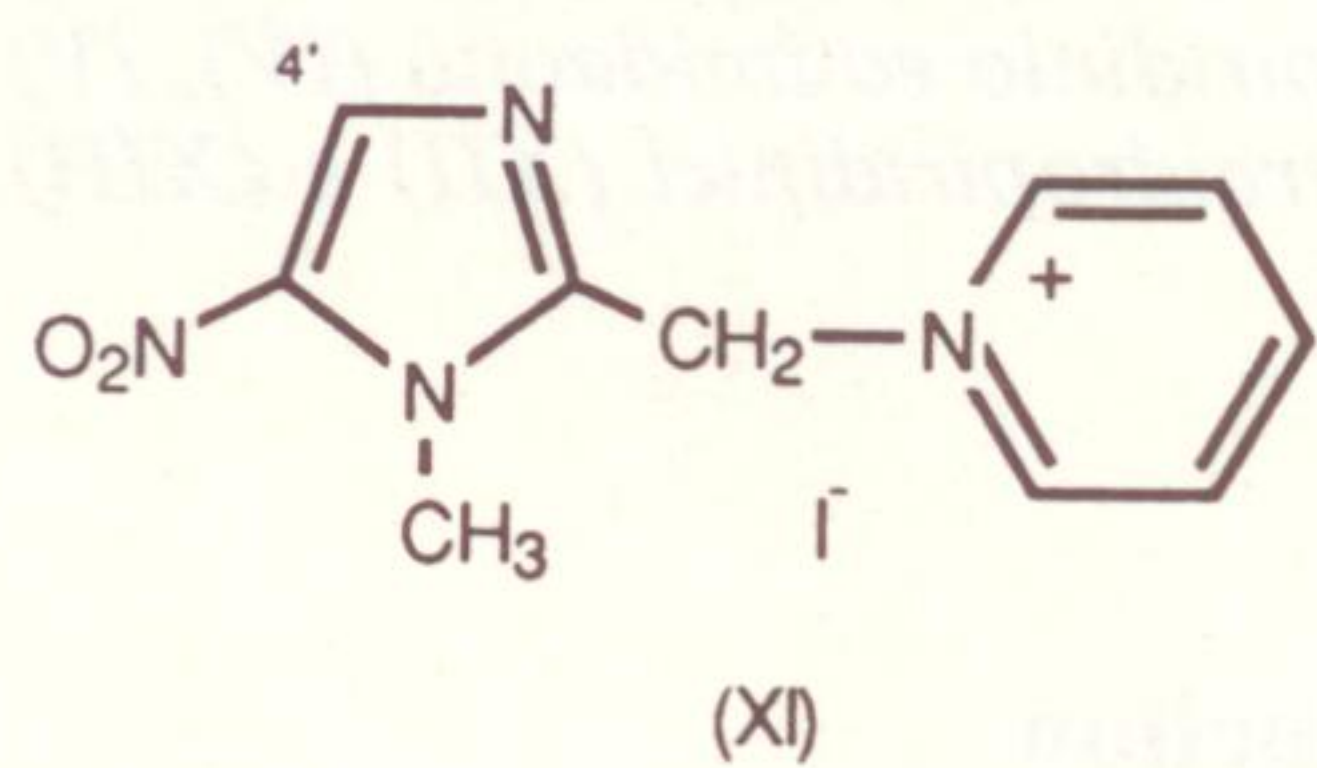
The 5-Nitroimidazoles derivatives (III) - (XIV) studied here belong to five different structures:



- (III) R = H
 (IV) R = (CH₃)₃-2,4,6
 (V) R = *t*-C₄H₉-4
 (VI) R = (C₆H₅)₃-2,4,6
 (VII) R = 3-CONH₂



- (VIII) R = CH₃
 (IX) R = *p*-C₄H₉
 (X) R = CH₂C₆H₅



- (XII) R = H
 (XIII) R = *t*-C₄H₉

The quaternary pyridinium salts (III) - (VII) and imidazolium salt (VIII) have been described previously (3). In a similar manner, reaction of 1-(2-chloroethyl)-2-methyl-5-nitroimidazole with 1-*n*-butylimidazole and 1-benzylimidazole afforded the more lipophilic imidazolium salts (IX) and (X) in good yields. On the other hand, compound (XI) was prepared from dimetridazole following Albright and Shephard (4). Treatment of the pyridinium salts (III), (V) and (XI) with sodium borohydride in alcohol gave the corresponding tetrahydropyridines (XII), (XIII) and (XIV).

The structures of the compounds were established according to their analytical and spectroscopic data. The ^1H and ^{13}C -N.M.R. spectra of compounds (III)-(VIII) have already been reported (3) and the remaining quaternary salts (IX), (X), (XI) do not deserve other comments (see Experimental). The ^1H and ^{13}C -N.M.R. chemical shifts of the tetrahydropyridines (XII), (XIII) and (XIV) are given in Tables I and II, and they have been assigned by comparison with data from other 1,2,5,6-tetrahydropyridines (5,6). The effects on the ^{13}C chemical shifts (see Table II) have shown that the protonation takes place on N(1').

Experimental section

A) CHEMISTRY

Melting points: on a CTP-MP 300 hot-plate apparatus (uncorr). I.R. spectra: Perkin-Elmer 1430 spectrophotometer. ^1H -N.M.R. spectra: at 200 MHz and ^{13}C -N.M.R. spectra: at 50.6 MHz on a Varian XL-200 spectrometer with chemical-shift values reported in δ (parts per million) relative to TMS as internal standard. Satisfactory analytical data ($\pm 0.4\%$ for C, H, N) were obtained for all compounds.

3-Butyl-1-[2-(2-methyl-5-nitro-1-imidazolyl)ethyl]imidazolium chloride (IX)

A mixture of 0.95 g (5 mmol) of 1-(2-chloroethyl)-2-methyl-5-nitroimidazole (3) and 1.86 g (15 mmol) of 1-*n*-butylimidazole (7) in 3 ml of DMF was refluxed for 48 h. After cooling, ether was added and an oil separated. It was triturated with anhydrous ether-HCl but the precipitate was hygroscopic and 1.41 g (95%) of (IX) was obtained as an oil. This sample was used for biological tests.

^1H -N.M.R. (DMSO- d_6): δ (p.p.m.) = 2.13 (s, 3H, CH_3 -2'), 8.07 (s, 1H, H-4'), 4.70 (t, 2H, CH_2 -N'), 4.74 (t, 2H, CH_2 -N), 9.26 (s, 1H, H-2), 7.74 (d, 1H, H-4), 7.82 (d, 1H, H-5), 4.15 (t, 2H, CH_2 -Pr), 1.70 (m, 2H, CH_2 -Et), 1.18 (m, 2H, CH_2 -Me), 0.87 (t, 3H, CH_3).

^{13}C -N.M.R. (DMSO- d_6): δ (p.p.m.) = 13.7 (CH_3 -2'), 151.5 (C-2'), 133.4 (C-4'), 138.5 (C-5'), 45.9 (CH_2 -N'), 48.3 (CH_2 -N), 137.0 (C-2), 122.9 (C-4), 123.1 (C-5), 48.8, 31.5, 18.8 and 13.3 (CH_2 - CH_2 - CH_2 - CH_3).

1-Benzyl-3-[2-(2-methyl-5-nitro-1-imidazolyl)ethyl]imidazolium chloride (X)

A mixture of 1.25 g (6.6 mmol) of 1-(2-chloroethyl)-2-methyl-5-nitroimidazole (3) and 3 g (19.8 mmol) of 1-benzylimidazole (7) in 4 ml of DMF was heated at 120°C for 52 h. After cooling, ether was added and an oil separated. The excess of 1-benzylimidazole was eliminated by distillation under vacuum. The residual oil was

Table I. ^1H NMR spectra of tetrahydropyridine derivatives

Compound	H-4'	Me-2'	CH ₂ -N'	CH ₂ -N	2H-2	H-3	H-4	2H-5	2H-6	R
(XII)(a)	7.97	2.44	4.38	2.68	2.78	5.60-5.63	1.92	2.51	---	---
(XIII)(b)	7.71	2.41	4.28	2.61	2.95	5.21	2.11	2.51	1.03	---
(XII)(b)	7.76	---	---	3.63	2.90	5.58	2.10	2.53	---	---

(a) Free base in DMSO-d₆; (b) Free base in CDCl₃;

Table II. ^{13}C NMR spectra of tetrahydropyridine derivatives

Compound	C-2'	C-4'	C-5'	Me-2'	CH ₂ -N'	CH ₂ -N	C-2	C-3	C-4	C-5	C-6	R
(XII)(a)	151.6	133.0	138.6	14.0	43.8	49.8	57.2	125.5	125.0	25.9	52.8	---
(XII)(b)	150.9	131.1	138.2	13.5	48.6	48.6	52.0	120.0	125.3	21.8	49.4	---
(XIII)(b)	151.3	131.5	138.4	13.8	48.5	48.5	50.0	110.8	144.4	21.5	49.7	35.0(C); 28.4(Me)
(XIV)(b)	143.4	132.0	139.8	---	---	48.8*	49.6	120.0	125.4	34.6	48.2*	---

(a) Free base in DMSO-d₆; (b) Hydrochloride in DMSO-d₆

* These signals could be interverted

dissolved in water and extracted with ether. The aqueous layer was evaporated to dryness and the oily residue triturated with anhydrous ether-HCl but the precipitate was hygroscopic and 1.65 g (71%) of compound (X) was obtained as an oil. This sample was used for biological tests.

¹H-N.M.R. (DMSO-d₆): δ (p.p.m.) = 2.02 (s, 3H, CH₃-2'), 8.05 (s, 1H, H-4'), 4.76 [m, 4H, (CH₂)₂], 9.54 (s, 1H, H-2), 7.83 (d, 1H, H-4), 7.92 (d, 1H, H-5), 5.48 (s, 2H, CH₂-Ph), 7.40 (m, 5H, Ph).

¹³C-N.M.R. (DMSO-d₆): δ (p.p.m.) = 13.5 (Me-2'), 151.4 (C-2'), 133.6 (C-4'), 138.6 (C-5'), 46.0 (CH₂-N'), 48.5 (CH₂-N), 137.1 (C-2), 123.0 (C-4), 123.6 (C-5), 52.2 (CH₂-Ph), 134.8, 128.5, 129.2 and 128.9 (aromat.).

1-(1-Methyl-5-nitro-2-imidazolylmethyl)pyridinium iodide (XI)

Obtained according to the literature (4) with the same yield and identical melting point.

¹H-N.M.R. (DMSO-d₆): δ (p.p.m.) = 4.03 (s, 3H, CH₃), 8.03 (s, 1H, H-4), 6.30 (s, 2H, CH₂), 8.28, 8.77 and 9.18 (5H, pyridinium).

¹³C-N.M.R. (DMSO-d₆): δ (p.p.m.) = 33.5 (CH₃), 145.2 (C-2'), 131.8 (C-4'), 139.6 (C-5'), 55.7 (CH₂), 145.5 (C-2, C-6), 128.2 (C-3, C-5) and 147.1 (C-4).

1-[2-(2-Methyl-5-nitro-2-imidazolyl)ethyl]1,2,5,6-tetrahydropyridine (XII)

To an ice-bath cooled solution of 1.46 g (5.4 mmol) of (III) in 70 ml of methanol, 0.40 g (10.6 mmol) of sodium borohydride was added portionwise. The resulting solution was refluxed for 8 h, then the solvent was removed at reduced pressure and 70 ml of water was added. The aqueous solution was continuously extracted with chloroform. Evaporation of the dried chloroform solution yielded 0.87 g (91%) of an oily (XII) was treated with ethanol saturated with HCl. The precipitate, the monohydrochloride of (XII) melted at 224-6°C. This sample was used for biological tests.

1-[2-(2-Methyl-5-nitro-1-imidazolyl)ethyl]-4-t-butyl-1,2,5,6-tetrahydropyridine (XIII)

Operating in the same manner as for the preparation of (XII) and starting from 1 g (3 mmol) of salt (V) and 0.23 g (6.1 mmol) of sodium borohydride in 30 ml of methanol, 0.84 g (93%) of compound (XIII), m.p. 107-110°C was obtained. Treatment of this compound with ethanol saturated with HCl yielded the corresponding monohydrochloride, m.p. 232-4°C.

1-(1-Methyl-5-nitro-2-imidazolylmethyl)-1,2,5,6-tetrahydropyridine (XIV)

With an identical experimental procedure as for (XII), 1.03 g (2.88 mmol) of salt (XI), 0.22 g (5.8 mmol) of sodium borohydride in 40 ml of methanol, yielded, after 64 h reflux, 0.53 g (80%) of (XIV) as an oil. This sample was used for biological tests.

B) BIOLOGY

Antitrichomoniasis activity

The strains of *Trichomonas vaginalis* used as an experimental model were cultured in Diamond medium without agar. The compounds to be tested, at the different pre-established doses, were added to the culture six hours after reseeded (hour

0); counts were made after 48 hours' contact of the compounds with the protozoa, using an automatic counting system by means of hematological meter (8). For each experiment, there were parallel growth control tubes, and also activity control tubes, using metronidazole as reference drug. Finally, the MIC (Minimum Inhibitory Concentration) and MCC (Minimum Cytocide Concentration) were calculated following the Howes procedure (9). Table III reports the results obtained.

Amebicidal activity

Entamoeba invadens in Standard Difco Entamoeba rice-starch complemented medium was used following a similar procedure as described for *T. vaginalis* (see paragraph above). However due to the large amount of starch particles present in the culture medium, it was not possible to use the automatic counting system that was substituted by the classic Neubauerhemocytometer microscopical countings. MIC and MCC were calculated according to Howes procedure (9) and taking metronidazole as the reference drug. Results are summarized in Table IV.

Antimalarial activity

The antipaludic activity of the different compounds was determined *in vivo*, using *Plasmodium berghei* as the experimental model. The method consists in the intraperitoneal inoculation of 10^6 parasited red cells in 20-25 g bw NMRI mice. The animals were arranged in homogeneous lots of not less than six animals each. The test molecules, suspended in carboxymethylcellulose, were administered by drench at 0, 1, 2 and 3 day post infection (p.i.). On the 5th day p.i. a blood smear from the tail of each mouse was made, in order to calculate the parasitation index of each animal. This allowed the calculation of the relationships between the untreated control lot and each of the treated lots. Finally, a statistical analysis of the results was made, calculating the Student's t, in order to find the significance in reduction, if any. Chloroquine was used as the reference drug, following the same procedure as with the nitroimidazole lots (Table V).

Antitrypanosomal activity

The compounds were assayed against epimastigote forms of *Trypanosoma cruzi* cultured at 28°C in LTM (Liquid Trypanosoma Medium) supplemented with 10% of Inactivated Foetal Bovine Serum (10). From exponential growing cultures, 100 μ l samples were taken and placed into 96 well microtiter plates, adjusted to 5×10^5 flagellates/ml. Dimethylsulfoxide solutions of the test compounds were made at 100, 10 and 1 μ g/ml final concentrations. The concentration of the solvent (DMSO) was about 0.2%, i.e. a concentration not toxic for the parasites. The antiparasitic activity tests were realized at 24, 48 and 72 hours after addition of the different products. Cell growth was measured by a colorimetric method (11) using a Kontron STL 210 spectrofotometer.

Nifurtimox was used as reference drug, following the same procedure as for the assayed compounds. In addition, the cytopathic effect on HeLa cells was evaluated using the same concentrations of the derivatives that were utilized against the parasites.

Anthelmintic activity

Compound (X) was tested *in vivo* against the tapeworm *Hymenolepis nana* and the roundworm *Syphacia obvelata*. Mice naturally infected with both parasites were coprologically selected from the animal house, and treated at the daily doses of 32

mg/kg/ bw for three consecutive days. After the treatment the mice were sacrificed and their intestines removed. Efficacy against *Hymenolepis nana* was corroborated after the dissection and removed the intestine contain in PBS solution. Compound (X) showed an effective reduction of 30% against *Hymenolepis nana* (30% of the treated animals were cured, with no eggs or intestinal adult worms upon dissection). The efficacy of this molecule against *Syphacia obvelata* was greater reaching 50% of the treated animals.

Antigiardic activity

Compounds (IV), (VI) and (X) were tested against *Giardia muris*. The mice infected with the parasite were treated orally with a dose of 32 mg/Kg during five days. Compounds (VI) and (X) led to parasite elimination in 30% of the treated animals. Similar results were obtained for metronidazole (the control drug).

Antifungal activity

Candida albicans (strain 1001) preserved in YED medium was used as an experimental model for antifungal screening. Activity was determined in SAH medium (Sabraud's agar-honey with chloramphenicol) and in NUCLON plates with 24 flat-bottom cups. 1 ml of medium was added to each cup and the plates thus prepared, once dry, were kept at 4°C until the time of the experiment. For the test, a culture of yeasts in SAH medium incubated 20 hours at 37°C was used, since at that moment the fungi were at the logarithmic stage of growth. Using this culture, a sterile saline solution was prepared so that the final concentration was 10⁵ yeasts/ml (12). Each cup was seeded with 10 µl of this suspension. Half an hour after seeding, the products to be tested were added at the pre-established dosage, dissolved in a maximum of 25 µl of DMSO. Amphotericine B was used as reference drug, using also DMSO as solvent. The nitroimidazoles were tested at initial dosages of 1600 µg; that dose was reduced only for compound (IV), in order to find the MIC (24 hours, µg/ml): for product (VI) it was >800 and for Amphotericine B it was 0.34.

Results and discussion

The antitrichomoniasic, amebicidal and antimalarial activities of the compounds were evaluated and the results are given in Tables III, IV and V. None of the compounds showed any antifungal activity against *Candida albicans* except product (IV).

Practically all the products were ineffective against epimastigote forms of the parasites except compounds (VI) and (X), which appeared to affect these organisms, but not HeLa cells. Compound (VI) produces a 68% inhibition of growth of epimastigote forms after 48 hours of culture at concentrations of 100 µg/ml. Compound (X) showed 18.4% of growth inhibition after 48 hours, at 100 µg/ml.

Studies of the epimastigote morphology revealed alterations in the typical parasite form. These altered cells appeared like rounded elements although motility remained normal. On the other hand, when epimastigote forms of *Trypanosoma cruzi* in exponential growth were cultured in Grace

Table IV. Amebicidal activity

Compound	Metronidazole	(III)	(IV)	(V)	(VI)	(VII)	(VIII)	(XI)	(XII)	(XIII)
MIC µg/mL	25	>400	>400	>400	>400	>400	>400	>400	200	100-200
MCC µg/mL	50-100	>400	>400	>400	>400	>400	>400	>400	400	100-200

Table III. Antitrichomoniasis activity

Compound	Metronidazole	(III)	(IV)	(V)	(VI)	(VII)	(VIII)	(XI)	(XII)	(XIII)
MIC µg/mL	1-2	>400	100	100-200	>400	>400	200	200-400	25-50	25-50
MCC µg/mL	2-4	>400	200-400	200	>400	>400	>400	>400	50	25-50

Table V Antimalarial activity^a

Compound	Chloroquine 10 mg/Kg	(III)	(IV)	(V)	(VI)	(VII)	(VIII)	(X)	(XI)	(XII)
%reduction	100%	0	50%	42% ^b	0	0	0	0	0	0

^a Nitroimidazoles tested at 100 mg/Kg; ^b Not significant (Student test).

Table VI . Biological results obtained with the 5-nitroimidazole derivatives. ^a

Compound	Trichomonas vaginalis	Entamoeba invadens	Plasmodium berghei	Trypanosoma cruzi	Hymenolepis nana	Syphacia obvelata	Giardia muris	Candida albicans
	In vitro Metronidazole	In vitro Metronidazole	In vitro Chloroquine	In vitro Allopurinol	In vivo Niclosamide	In vivo Pirantel pamoate	In vivo Metronidazole	In vitro Anfotericine B
(III)	0	0	0	0	---	---	---	0
(IV)	**	0	***	0	---	---	**	*
(V)	**	0	*	0	---	---	---	0
(VI)	0	0	0	**	---	---	0	0
(VII)	0	0	0	0	---	---	---	0
(VIII)	*	0	0	0	---	---	---	0
(IX)	---	---	---	0	---	---	---	---
(X)	---	---	---	***	***	0	**	---
(XI)	*	0	0	0	---	---	---	0
(XII)	***	**	0	0	---	---	---	---
(XIII)	***	***	0	0	---	---	---	---
(XIV)	---	---	---	0	---	---	---	---

^aThe stars represent relative potencies of the compounds (III)-(XIV); the reference compounds are always more active

medium (Grace's insect medium, GIBCO) supplemented with 10% IFCS (13), no morphological alterations were observed. Inhibition percentage (14) after 72 hours was about 40% for (VI) and 53.8% for (X). These differences might be the consequence of a higher metabolic parasitic activity in the Grace modified medium (Normally, this medium is employed for mass production of epimastigote forms).

Liquid scintillation measurements of the incorporation of [5-³H] uridine indicated a decrease in RNA synthesis after 135 min incubation with products (VI) and (X), while incorporation of [4-5-³H] leucine and [6-³H] thymidine (protein and DNA synthesis) remained unaltered.

Compound (X) was tested *in vivo* against *Hymenolepis nana* and *Syphacia obvelata*. Compounds (IV), (VI) and (X) were tested against *Giardia muris*. The results obtained are reported in the biological section.

All the compounds were devoid of cytotoxicity (when tested against HeLa cells). Eight different parasites were studied, *in vitro* and *in vivo* using different drugs as standards and Table VI summarized the results obtained. Among the quaternary salts, the compounds showing some activity were the less hydrophilic derivatives (IV), (V), and (X). Compound (VI) is probably sterically hindered even if it is one amongst the least hydrophilic. The tetrahydropyridines (XII) and (XIII) improved considerably their antitrichomoniasic and amebicidal activities. This clearly indicates that the quaternary ammonium salt structure of compounds (III)-(XI) is too hydrophilic to adequately penetrate the cellular wall of the parasite.

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