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Synthesis and Antimicrobial Activity of New 3-(1-*R*-3(5)-Methyl-4-nitroso-1*H*-5(3)-pyrazolyl)-5-methylisoxazoles

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Abstract—A number of new 3-(1-*R*-3(5)-methyl-4-nitroso-1*H*-5(3)-pyrazolyl)-5-methylisoxazoles **6a–g** (**7b–f**) were synthesized and tested for antibacterial and antifungal activity. Some of these compounds displayed antifungal activity at non-cytotoxic concentrations. Derivative **6c** was 9 times more potent in vitro than miconazole and 20 times more selective against *C. neoformans*. **6c** was also 8- and 125-fold more potent than amphotericin B and fluconazole, respectively. None of the compounds was active against bacteria. Preliminary structure–activity relationship (SAR) studies showed that the NO group at position 4 of the pyrazole ring is essential for the activity. Lipophilicity of the pyrazole moiety, *N*-alkyl chain length and planarity of the two heterocyclic rings appear to play a decisive role in modulating cytotoxicity and antifungal activity.

Introduction

Cryptococcosis is a disseminated infection caused by the ubiquitous yeast *Cryptococcus neoformans*.[1] Since this fungus is primarily a pathogen for immunocompromised patients, individuals with AIDS are at high risk of cryptococcosis. In the setting of AIDS, cryptococcal infections are particularly difficult to treat because antifungal therapy does not usually eradicate the infection. Given the high incidence of relapse after initial antifungal therapy, current management of *C. neoformans* infections in patients with AIDS includes lifelong suppressive therapy with antifungal drugs. Therefore, the need for novel antifungal agents for cryptococcosis and other opportunistic infections is apparent in light of the significant problems associated with current drugs and makes the development of new drug entities all the more urgent.

In our previous papers, we reported that some nitrosopyrazolyl-isoxazoles inhibited the growth of the fungus *M. canis* at 6.25 μg/mL.[2] Moreover, the occurrence of 4-nitrosopyrazoles of biological interest is indicated by the large number of reports dealing with their numerous biological activities,[3–10] but despite the existing interest on this class of compounds, virtually nothing is known about the possible structure–activity relationships (SARs).

These findings and the increased interest on new antifungal agents led us to synthesize, to define the structures of title compounds and to test in vitro their antifungal properties in order to identify the structural features essential for the activity of 4-nitrosopyrazoles.

Chemistry

Derivatives **4b–d**, **5b–d**, **6a–g** and **7b–f** were prepared by reaction of β-diketones **1** and **2** with suitable commercial hydrazino derivatives **3a–g** according to modified methods previously reported by us.[2] The synthetic pathway (Scheme 1) allows the formation of a mixture of two isomers that were easily separated by ordinary silica gel column chromatography. Yields, mp, selected reaction conditions to obtain the two isomeric series in

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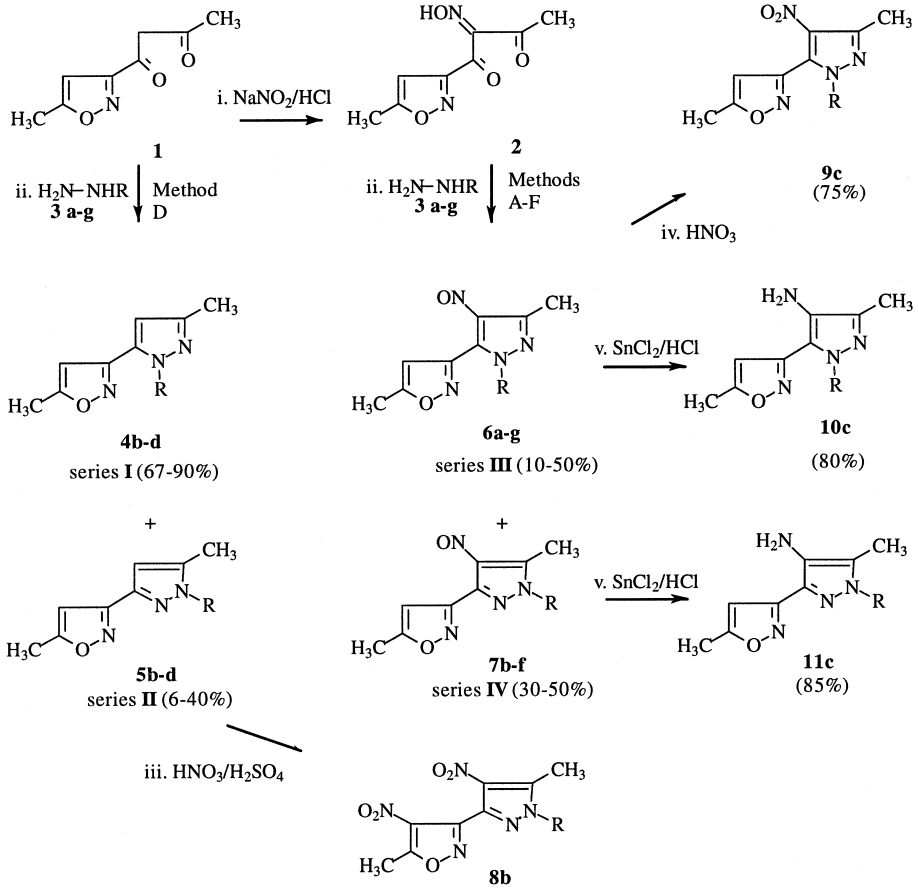


Scheme 1. (i) $NaNO_2$/HCl in water at rt; (ii) method A: water at rt; B: dichloromethane at rt for 10 min; C ethanol at 0 °C to rt; D: ethanol at reflux for 1 h; E: water/OH^- at rt; F: acetic acid at reflux for 1 h; (iii) H_2SO_4/HNO_3 at rt for 3 h; (iv) HNO_3 on ice bath in 5 h; (v) $SnCl_2$/HCl concd at 0 °C.

good yields, *m/z*, IR and ^{1}H NMR spectral data are given in Tables 1 and 2.

The reaction of **2** with **3d** (R = CH_2CH_2OH) in water produced **7d** (80%) and **6d** (10%) after 3 days. Similar yields were obtained in dichloromethane, but in this case the reaction was completed in just 10 min. When the reaction was initially performed in absolute ethanol at 0 °C and then kept at room temperature for 3 days both isomers in comparable yields were obtained. Reaction of **2** with phenylhydrazine, carried out in several different experimental conditions, gave only the isomer **6g** in low yields (30% in refluxing acetic acid and 20% in refluxing ethanol). Compound **9c** was easily obtained by oxidation of **6c** with nitric acid at 5 °C. Finally, reduction of **6c** carried out in concentrated hydrochloric acid and stannous choride afforded in good yields compound **10c**.

Owing to the availability of both **III** and **IV** pure isomeric series, the chemical structure of all derivatives was elucidated by elemental analysis, IR, ^{1}H NMR, ^{13}C NMR and X-ray diffractometric data.

The experimental ^{13}C NMR spectral data are listed in Table 3. The interpretation of ^{13}C NMR spectra of isomeric *N*-alkylated azoles was made by considering that a carbon adjacent to an alkylated nitrogen resonates upfield of the signal of the same carbon in the other isomer.[11] In fact the ^{13}C NMR signals of C-5 **4b–d** derivatives were upfield with respect to the signals of the corresponding C-3 **5b–d** isomers. In the same manner the C-3 and C-5 signals relative to the compounds of the series **I** and **II** were assigned according to literature data.[12]

With regard to the series of compounds **III** and **IV**, the presence of a nitroso substituent at position 4′ involves a high degree of chemical shielding anisotropy.[13,14] In the case of **6e** and **7e**, the chemical shifts were 159.21 and 158.78 ppm, respectively.

The singlet which appeared as the most deshielded one was assigned to C-5 and the less shielded one to C-3. The only doublet observed at low field for nitroso derivatives **6b–g** and **7b–f** was assigned by DEPT to C-4.

In all derivatives, signals observed between 11.62 and 11.80 ppm, not showing significant chemical shift variations, were assigned to the isolated 5-methyl carbon.

The signals attributed to 5-methyl carbon of **7b–f** derivatives consistantly resonate upfield with respect to the 3′-methyl carbon of compounds **6b–g**: the observed

Table 1. Yields, physical and spectroscopic data of series **I** and **II**

Compound	Mp (°C)[d]	Eluant[e]	Yield (%)[f]	*m/z*	Molecular formula	IR (cm^{-1})	^1H NMR (δ, ppm), (DMSO-d_6)
4b[a]	35 (c)	(1)	67	177	$C_9H_{11}N_3O$		2.19 (3H, s, CH_3), 2.47 (3H, s, CH_3), 4.00 (3H, s, CH_3), 6.55 (1H, s, CH), 6.63 (1H, s, CH)
4c[b]	57 (c)	(1)	40	191	$C_{10}H_{13}N_3O$		1.32 (3H, t, $J=6.8$ Hz, CH_3), 2.21 (3H, s, CH_3), 2.47 (3H, s, CH_3), 4.40 (2H, q, $J=6.8$ Hz,CH_2), 6.55 (1H, s, CH), 6.62 (1H, s, CH)
4d[c]	87 (b)	(3)	90	207	$C_{10}H_{13}N_3O_2$	3320 (OH, broad)	2.22 (3H, s, CH_3), 2.47 (3H, s, CH_3), 3.78 (2H, q, $J=5.8$ Hz, CH_2), 4.46 (2H, t, $J=5.1$ Hz, CH_2), 4.91 (1H, t, $J=5.1$ Hz, OH), 6.51 (1H, s, CH), 6.60 (1H, s, CH)
5b[a]	125–126 (c)	(1)	11	177	$C_9H_{11}N_3O$		2.28 (3H, s, CH_3), 2.42 (3H, s, CH_3), 3.78 (3H, s, CH_3), 6.44 (1H, s, CH), 6.46 (1H, s, CH)
5c[b]	72 (c)	(2)	40	191	$C_{10}H_{13}N_3O$		1.33 (3H, t, $J=7.3$ Hz, CH_3), 2.30 (3H, s, CH_3), 2.42 (3H, s, CH_3), 4.10 (2H, q, $J=7.3$ Hz, CH_2), 6.44 (1H, s, CH), 6.48 (1H, s, CH)
5d[c]	88 (b)	(3)	6	207	$C_{10}H_{13}N_3O_2$	3395 (OH, broad)	2.31 (3H, s, CH_3), 2.42 (3H, s, CH_3), 3.72 (2H, q, $J=5.8$ Hz, CH_2), 4.11 (2H, t, $J=5.8$ Hz,CH_2), 4.90 (1H, t, $J=5.8$ Hz, OH), 6.41 (1H, s, CH) 6.47 (1H, s, CH)

[a]R = Me.
[b]R = Et.
[c]R = CH_2CH_2OH.
[d]Crystallization solvent: (c) = cyclohexane, (b) = benzene.
[e]Chromatographic eluant: (1) = petroleum ether 40–60°: ethyl acetate 1:1; (2) = petroleum ether 40–60°: ethyl acetate 7:3; (3) = dichloromethane:ethyl acetate 7:3.
[f]Refluxed in ethanol for 1 h.

signal pattern is justified, as mentioned above, by the effects of the alkyl group on the nearest nitrogen atom.[11,15]

Because of the nitroso group rotation,[16] the C-3′ and C-5′ carbon signals, registered at room temperature at 50.3 MHz, appeared as two broad and weak peaks at the shift values listed in Table 3. These assignments were supported by structural X-ray analysis assuming a good structure correspondence between the solution and the solid state,[14] in which the NO group assumes a stable conformation.

Due to the availability of only one isomer, the carbon signals of **6g** were assigned on the basis of an analogous signal pattern of compounds belonging to series **III**.

Additional evidence for the above assignments came from the ^1H NMR spectra. The 3-CH_3 protons of compounds **6b**–**g** appeared as a singlet downfield with respect to the 5-CH_3 protons of compounds **7b**–**f**, according to the electronic and steric effects of substituents situated on the vicinal nitrogen atom.[11]

With regard to IR spectra, all **6a**–**g** and **7b**–**f** nitroso derivatives showed a characteristic and strong NO absorption band in the range 1327–1372 cm^{-1}. In the series **7b**–**f**, the frequency value of the absorption band was slightly higher than the value found for the **6a**–**g** series having the same *N*-alkyl group (see Table 2).

X-ray diffraction

Since it was possible to produce well formed crystals of **6e** and **7e** by recrystallization from dilute ethanolic solution we were able to obtain crystal data and structure refinement for these two compounds. The results provided useful data for preliminary SAR studies.

Biology

Compounds were tested for antimicrobial activity against representative human pathogenic fungi (*C. albicans, C. parapsilosis, C. paratropicalis, C. kruzei, C. glabrata, C. laurentii, C. neoformans, A. fumigatus, T. mentagrophytes, T. capitatum*), against Gram-negative (*Shigella* spp., *Salmonella* spp.) and Gram-positive bacteria (*Staphylococcus aureus,* group D *Streptococcus*). Test compounds were also evaluated for antiretroviral activity in MT-4 cells infected with HIV-1. Cytotoxicity against MT-4 cells, carried out in parallel with anti-HIV-1 activity, was evaluated to determine whether the compounds were endowed with selective antimicrobial/antiviral activity.

Miconazole, fluconazole, and amphotericin B were used as reference compounds in antimycotical assays. Streptomycin and AZT were used in antibacterial and antiretroviral assays, respectively.

Results and Discussion

Test compounds showed inhibitory activity against fungi at concentrations ranging between 0.1 and 200 μM (MIC/MFC in Table 4). Derivatives **6b**–**e** were active against *C. neoformans* at concentrations equal to or lower than those of miconazole (MIC and MFC, 0.9 μM). Due to a lower cytotoxicity for MT-4 cells (CC_{50} = 42 μM), compound **6c** was 9 times more potent and 20-fold more selective than miconazole, **6c** was also 8- and 125-fold more potent than amphotericin B and

Table 2. Yields, physical and spectroscopic data of series **III** and **IV**

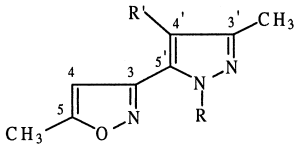
Compound	Mp (°C)[h]	Eluant[i]	Yield (%)/ method[k]	*m/z*	Molecular formula	IR (cm^{-1})	^1H NMR (δ ppm), (DMSO-d_6)
6a[a]	188 (b) (1)	(4)	50/A	192	$C_8H_8N_4O_2$	1327 (NO) 3170 (NH)	2.37 (3H, s, CH_3), 2.55 (3H, s, CH_3), 6.80 (1H, s, CH), 12.98 (1H, broad NH)
6b[b]	57 (e) (1)	(1)	30/C [j]/E	206	$C_9H_{10}N_4O_2$	1334 (NO, strong)	2.12 (3H, s, CH_3), 2.59 (3H, s, CH_3), 4.09 (3H, s, CH_3), 7.03 (1H, s, CH)
6c[c]	107 (e) (2)	(5)	35/A 30/E	220	$C_{10}H_{12}N_4O_2$	1337 (NO, strong)	1.45 (3H, t, $J=7.3$ Hz, CH_3), 2.15 (3H, s, CH_3), 2.60 (3H, s, CH_3), 4.46 (2H, q, $J=7.3$ Hz, CH_2), 7.02 (1H, s, CH)
6d[d]	148 (e) (2)	(6)	10/A 10/B 30/C	236	$C_{10}H_{12}N_4O_3$	1342 (NO, strong) 3440 (OH, broad)	2.16 (3H, s, CH_3), 2.60 (3H, s, CH_3), 3.84 (2H, t, $J=5.8$ Hz, CH_2), 4.51 (2H, t, $J=5.8$ Hz, CH_2), 5.00 (1H, t, $J=5.8$ Hz, OH), 7.00 (1H, s, CH)
6e[e]	67–68 (e) (3)	(7)	50/A	234	$C_{11}H_{14}N_4O_2$	1341 (NO, strong)	0.90 (3H, t, $J=7.3$ Hz, CH_3), 1.89 (2H, m, $J=7.3$ Hz, CH_2), 2.15 (3H, s, CH_3), 2.60 (3H, s, CH_3), 4.40 (2H, t, $J=7.3$ Hz, CH_2), 7.02 (1H, s, CH)
6f[f]	48–49 (e–w) (2)	(5)	45/A 30/E	248	$C_{12}H_{16}N_4O_2$	1341 (NO, strong)	0.90 (3H, t, $J=7.3$ Hz, CH_3), 1.33 (2H, m, $J=7.3$ Hz, CH_2), 1.85 (2H, m, $J=7.3$ Hz, CH_2), 2.14 (3H, s, CH_3), 2.60 (3H, s, CH_3), 4.45 (2H, t, $J=7.3$ Hz, CH_2), 7.02 (1H, s, CH)
6g[g]	120–121 (e) (2)	(8)	20/D 30/F	268	$C_{14}H_{12}N_4O_2$	1346 (NO, strong)	2.26 (3H, s, CH_3), 2.51 (3H, s, CH_3), 6.76 (1H, s, CH), 7.55 (1H, s, C_6H_5)
7b[b]	72 (e) (3)	(2)	40/C [j]/E	206	$C_9H_{10}N_4O_2$	1348 (NO, strong)	2.52 (3H, s, $CH_3\times2$), 3.88 (3H, s, CH_3), 6.69 (1H, s, CH)
7c[c]	60 (e) (3)	(5)	50/A 45/E	220	$C_{10}H_{12}N_4O_2$	1345 (NO, strong)	1.4 (3H, t, $J=7.3$ Hz, CH_3), 2.53 (3H, s, CH_3), 2.56 (3H, s, CH_3), 4.23 (2H, q, $J=7.3$ Hz, CH_2), 6.69 (1H, s, CH)
7d[d]	146–147 (e) (3)	(5)	80/A 80/B 40/C	236	$C_{10}H_{12}N_4O_3$	1362 (NO, strong) 3468 (OH, broad)	2.53 (3H, s, CH_3), 2.59 (3H, s, CH_3), 3.84 (2H, t, $J=5.1$ Hz, CH_2), 4.27 (2H, t, $J=5.1$ Hz, CH_2), 5.07 (1H, t, $J=5.1$ Hz, OH), 6.69 (1H, s, CH)
7e[e]	58–59 (e) (3)	(7)	30/A	234	$C_{11}H_{14}N_4O_2$	1345 (NO, strong)	0.91 (3H, t, $J=7.3$ Hz, CH_3), (3H, s, CH_3), 1.84 (2H, m, $J=7.3$ Hz, CH_2), 2.54 (3H, s, CH_3), 2.57 (3H, s, CH_3), 4.17 (2H, t, $J=7.3$ Hz, CH_2), 6.70 (1H, s, CH)
7f[f]	52–53 (e–w)(3)	(5)	45/A 35/E	248	$C_{12}H_{16}N_4O_2$	1348 (NO, strong)	0.92 (3H, t, $J=7.3$ Hz, CH_3), 1.33 (2H, m, $J=7.3$ Hz, CH_2), 1.80 (2H, m, $J=7.3$ Hz, CH_2), 2.53 (3H, s, CH_3), 2.56 (3H, s, CH_3), 4.20 (2H, t, $J=7.3$ Hz, CH_2), 6.69 (1H, s, CH)
9c[c]	98–100 (e) (4)	(2)	75	236	$C_{10}H_{12}N_4O_3$	1516,1344 (NO_2)	1.32 (3H, t, $J=6.8$ Hz, CH_3), 2.50 (3H, s, CH_3), 2.56 (3H, s, CH_3), 4.07 (2H, q, $J=6.8$ Hz, CH_2), 6.68 (1H, s, CH)
10c[c]	Oil	(9)	80	206	$C_{10}H_{14}N_4O$	3412, 3342 (NH_2)	1.21 (3H, t, $J=6.8$ Hz, CH_3), 2.08 (3H, s, CH_3), 2.46 (3H, s, CH_3), 4.15 (2H, bs, NH_2), 4.19 (2H, q, $J=6.8$ Hz, CH_2), 6.68 (1H, s, CH)
11c[c]	118–119 (c) (5)	(3)	80	206	$C_{10}H_{14}N_4O$	3412, 3328 (NH_2)	1.28 (3H, t, $J=7.3$ Hz, CH_3), 2.16 (3H, s, CH_3), 2.42 (3H, s, CH_3), 4.01 (2H, q, $J=7.3$ Hz, CH_2), 4.20 (2H, bs, NH_2), 6.43 (1H, s, CH)

[a]**a** R = H.
[b]**b** R = Me.
[c]**c** R = Et.
[d]**d** R = CH_2CH_2OH.
[e]**e** R = *n*-Pr.
[f]**f** R = *n*-Bu.
[g]**g** R = Ph.
[h]Crystallization solvent: (b) benzene, (e) ethanol, (e–w) ethanol–water, (c) petroleum ether. Crystal appearance: (1) blue-green prism, (2) emerald-green prism, (3) blue-green needle, (4) white needle, (5) yellow needle.
[i]Chromatographic eluant: (1) = petroleum ether 40–60°:ethyl acetate 1:1; (2) = petroleum ether 40–60°:ethyl acetate 7:3; (3) = dichloromethane:ethyl acetate 7:3; (4) = dichloromethane:ethyl acetate 9:1; (5) = dichloromethane; (6) = diethyl ether:methanol 96:4; (7) = dichloromethane:petroleum ether 40–60° 6:4; (8) = petroleum ether 40–60°:diethyl ether 9:1; (9) = diethyl ether.
[j]Dark intractable rubber.
[k]Method: A water, rt; B dichloromethane, rt for 10 min; C ethanol at 0 °C to rt; D ethanol at reflux for 1 h; E water/OH^-, rt; F acetic acid at reflux for 1 h.

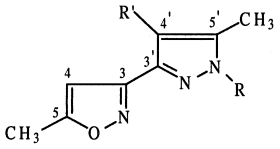
fluconazole, respectively, against *C. neoformans*. Compounds **6b–e** were also active against *Candida* spp., *A. fumigatus* and *T. mentagrophytes* at concentrations slightly higher than those of miconazole.

Derivative **6e** had a wider range of activity, with a potency comparable to that of miconazole against *C. albicans*, *C. paratropicalis*, *C. glabrata*, *C. neoformans* and *A. fumigatus*.

Table 3. ^{13}C NMR Chemical shifts registered at 50.3 MHz, in DMSO-d_6[a]



4b–d, R′ = H; **6a–g,** R′ = NO; **9c**, R = NO_2; **10c**, R = NH_2



5b–d, R′ = H; **7a–f,** R′ = NO; **11c**, R = NH_2

Compound	C-5	C-4	C-3	C-5	C-4	C-3	3-CH_3	5-CH_3	5-CH_3	N-C1	N-C2	N-C3	N-C4
4b	169.83	101.51	154.11	131.92	107.94	146.61	12.97		11.64	38.77			
4c	169.81	101.61	154.01	131.06	107.50	146.84	13.10		11.62	45.06	15.46		
4d	169.78	101.82	154.21	132.27	107.35	147.13	13.10		11.61	52.81	60.19		
5b	169.36	99.78	157.11	139.38	103.43	140.24		10.63	11.74	36.19			
5c	169.34	99.74	157.54	139.61	103.54	139.40		10.44	11.73	43.59	15.11		
5d	169.34	99.76	157.51	139.82	103.31	140.83		10.88	11.75	51.26	60.28		
6a	170.38	102.49	155.23	133.23	158.84	139.67	11.15		11.80				
6b	171.23	104.28	152.52	133.20	159.37	138.69	12.89		11.72	39.50			
6c	171.35	104.50	152.43	133.69	159.32	137.97	14.82		11.74	46.78	13.04		
6d	171.11	104.78	152.56	133.80	159.46	139.48	13.04		11.71	59.34	53.56		
6e	171.27	104.50	152.42	133.56	159.21	138.31	12.96		11.68	52.73	22.57	10.67	
6f	171.20	104.42	152.42	133.39	159.16	138.24	13.27		11.62	51.03	31.15	19.08	12.91
6g	171.02	104.53	152.20	135.24	159.59	138.03	12.86		11.68	139.06 (C-1-Ph); 129.54 (C-3,5-Ph); 125.54 (C-2-6-Ph); 129.24 (C-4-Ph)			
7b	170.13	102.40	155.36	135.11	158.88	136.78		10.06	11.73	36.33			
7c	170.10	102.46	155.43	134.74	158.89	136.77		9.81	11.71	43.95	14.11		
7d	170.16	102.53	155.49	136.77	159.01	138.81		10.31	11.75	59.47	51.60		
7e	170.09	102.46	155.42	134.98	158.78	137.03		9.98	11.70	50.12	22.10	10.70	
7f	170.08	102.45	155.40	134.75	158.79	137.00		9.94	11.69	48.46	30.67	19.14	13.37
9c	170.85	103.98	152.27	130.59	145.49	131.49	13.49		11.79	46.05	15.09		
10c	169.36	100.35	153.94	129.54	116.33	135.86	10.58		11.74	45.41	15.44		
11c	168.24	99.32	158.58	123.47	127.19	127.84		8.03	11.56	43.91	15.07		

[a] **a** R = H, **b** R = Me, **c** R = Et, **d** R = CH_2CH_2OH, **e** R = *n*-Pr, **f** R = *n*-Bu, **g** R = Ph.

Compounds of the series **I** and **II** and the derivative **8b** were noncytotoxic for MT-4 cells at doses as high as 200 μM, whereas compounds of the series **III** and **IV** showed a cytotoxicity comparable to that of miconazole (CC_{50} = 18 μM) with the exception made for derivative **7d**, which was 6-fold more toxic.

All derivatives were neither significantly active against Gram-negative and Gram-positive bacteria (Table 5), nor capable of protecting MT-4 cells from the cytopathic effect induced by HIV-1, at least at noncytotoxic concentrations. Under the same experimental conditions AZT was active at 0.01 μM.

The absence of the NO group (series **I** and **II**) or its replacement with the NO_2 group (**9c**) or with the amino group (**10c**) gave compounds devoid of antimycotical activity. The dinitro derivative **8b** was also inactive.

Only compounds belonging to the two series **III** and **IV** proved active as antifungal agents. Compounds **6a–c**, **6e–f** and **7b–f** displayed selective activity against some fungi (Table 4).

The potency of NO-carrying derivatives appeared to be modulated by the type of substituent and by the length of the carbon chain of the alkyl group bound to the nitrogen of the pyrazole ring: the N–H (**6a**) and *N*-phenyl (**6g**) derivatives were poorly active. With the increasing length of the alkylic carbon chain, the activity increases passing through a maximum with the ethyl group, decreasing progressively with the propyl and butyl groups.

Lipophilicity measurements (R_M) pointed out constantly higher R_M values for derivatives **6b–f** (series **III**) than for corresponding isomers **7b–f** (series **IV**). Compounds belonging to the series **III** were more active and less cytotoxic than isomeric 1*H*-3-pyrazolyl derivatives of series **IV**.

The trend of the observed activity can be correlated with data obtained in the determination of crystal structures of two isomeric compounds **6e** and **7e** (Figs 1 and 2). The nitroso group of **6e** adopts *trans* configuration with respect to the alkyl chain on the pyrazole nitrogen and extended perpendicularly to the average molecular plane, as already observed for related compounds both in solution and in the solid state.[14] On the contrary, the nitroso group of **7e** adopts *cis* configuration. The geometry of **6e** can be compared with the other two nitrosopyrazole compounds whose structures are known, 1 *H*-3,5-di-*tert*-butyl-4-nitrosopyrazole and 1,3,5-trimethyl-4-nitrosopyrazole.[14] These compounds present partial *cis*/*trans* disorder in the solid state, while **6e** is found in the pure *trans* form. This is probably due to the different steric hindrance of the 3-methyl and 4-isoxazole substituents, which favor the orientation of the NO group away from the latter. Moreover, in **6e** the

Table 4. Antifungal activity and R_M value

Compound	CC_{50}[a]	MIC[b]/MFC[c]										
	MT-4	*C. albicans*	*C. parapsilosis*	*C. paratropicalis*	*C. kruzei*	*C. glabrata*	*C. laurenti*	*C. neoformans*	*A. fumigatus*	*T. mentagrophytes*	*T. capitum*	R_M
4b	> 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	−0.158
5b	> 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	−0.286
4c	> 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	−0.084
5c	> 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	−0.203
4d	> 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	−0.357
5d	> 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	−0.434
6a	26	> 200/ > 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	30/60	> 200/ > 200	> 200/ > 200	> 200/ > 200	−0.277
6b	11.6	25/25	66/ > 200	25/ > 25	6/6	25/25	12/25	0.8/1.6	12/22	25/25	12/12	0.008
7b	9.1	> 200/ > 200	> 200/ > 200	200/200	25/50	100/100	50/50	3.1/3.1	22/66	66/66	25/100	−0.248
6c	42	19/19	9/9	19/19	30/60	19/19	9/19	0.1/0.1	9/9	19/19	75/ > 75	0.089
7c	9.5	66/66	> 200/ > 200	66/200	100/100	50/100	25/50	3.1/3.1	66/66	200/200	100/100	−0.149
6d	13.5	66/66	> 200/ > 200	66/66	66/66	66/66	ND	0.8/0.8	22/22	2.4/7.4	66/200	−0.194
7d	3.35	> 200/ > 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	22/22	> 200/ > 200	> 200/ > 200	> 200/ > 200	−0.360
6e	20	7.4/7.4	66/66	7.4/7.4	22/22	7.4/7.4	ND	0.8/0.8	7.4/7.4	2.4/7.4	66/66	0.163
7e	14	22/22	66/66	22/22	66/66	22/22	ND	2.4/2.4	7.4/7.4	2.4/7.4	66/66	−0.059
6f	15	22/22	66/200	22/22	66/66	22/200	ND	2.4/2.4	7.4/200	200/200	66/66	0.038
7f	92	> 200/ > 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	150/150	75/150	9/9	37/37	75/75	> 200/ > 200	0.278
6g	14	> 200/ > 200	200/200	> 200/ > 200	200/ > 200	> 200/ > 200	ND	66/66	66/200	66/200	200/ > 200	0.142
8b	> 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	0.078
9c	> 200	> 200	> 200	> 200	> 200	> 200	ND	> 200	ND	ND	ND	ND
10c	> 200	> 200	> 200	> 200	> 200	> 200	ND	> 200	ND	ND	ND	ND
-[d]	18	7/7	0.9/0.9	7/7	2/4	6/6	0.5/0.9	0.9/0.9	4/4	0.9/0.9	4/4	ND
-[e]	> 200	200/ > 200	200/200	12.5/ > 200	200/200	200/ > 200	ND	12.5/12.5	ND	ND	ND	ND
-[f]	> 200	3.1/3.1	0.8/3.1	0.2/3.1	12.5/12.5	3.1/3.1	ND	0.8/0.8	ND	ND	ND	ND

[a]Compound dose (μM) required to reduce the viability of mock-infected cells by 50%.
[b]Minimum inhibitory concentration.
[c]Minimum fungicidal concentration.
[d]Miconazole.
[e]Fluconazole.
[f]Amphotericin B.

Table 5. Antibacterial activity

Compound	CC_{50}[a]	MIC[b]/MBC[c] (μM)				
	MT-4	*Shigella*	*Salmonella*	*Staphylococcus*	*Streptococcus*	R_M[d]
4b	> 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	−0.158
5b	> 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	−0.286
4c	> 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	−0.084
5c	> 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	−0.203
4d	> 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	−0.357
5d	> 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	−0.434
6a	26	> 200/ > 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	−0.277
6b	11.6	200/ > 200	200/ > 200	200/ > 200	200/ > 200	0.008
7b	9.1	66/66	66/66	200/ > 200	200/ > 200	−0.248
6c	42	> 200/ > 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	0.089
7c	9.5	66/66	> 200/ > 200	200/ > 200	200/ > 200	−0.149
6d	13.5	100/100	100/200	100/100	100/100	−0.194
7d	3.35	22/66	66/66	22/66	22/66	−0.360
6e	20	> 200/ > 200	> 200/ > 200	200/200	200/200	0.163
7e	14	> 200/ > 200	> 200/ > 200	200/200	200/200	−0.059
6f	92	> 200/ > 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	0.278
7f	15	> 200/ > 200	> 200/ > 200	100/100	100/100	0.038
6g	14	> 200/ > 200	> 200/ > 200	50/100	50/50	0.142
8b	> 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	0.078
9c	> 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	ND
10c	> 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	> 200/ > 200	ND
Streptomycin	> 200	3.1/3.1	6.2/6.2	3.1/3.1	3.1/3.1	

[a]Compound dose (μM) required to reduce the viability of mock-infected cells by 50%.
[b]Minimum inhibitory concentration.
[c]Minimum bactericidal concentration.
[d]Higher R_M value correspond to higher lipophilicity.

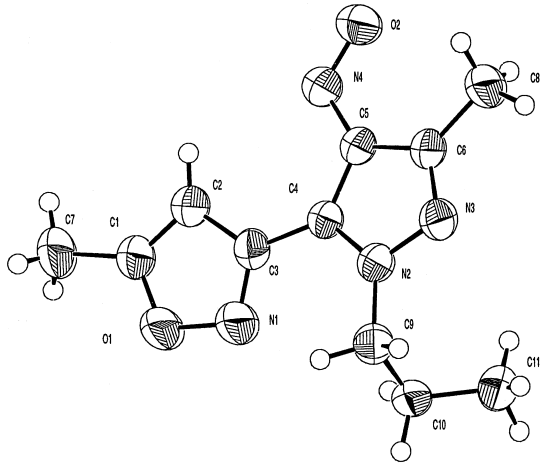


Figure 1. Perspective view of the molecular structure of **6e**. Thermal ellipsoids are drawn at the 50% probability level.

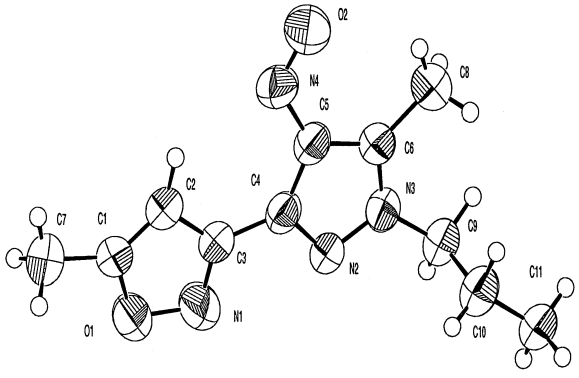


Figure 2. Perspective view of the molecular structure of **7e**. Thermal ellipsoids are drawn at the 50% probability level.

isoxazole and pyrazole rings, both planar within 0.03 Å, define a dihedral angle of 13.3° due to the intramolecular interaction between the propyl chain and the isoxazole ring. In the isomer **7e** the two rings, both again planar within 0.03 Å, define a dihedral angle of 2.7°, as the entire molecule is planar within 0.06 Å. In **7e** the nitroso group is *cis* with respect to the N3–C9 bond, that is, it prefers pointing towards the methyl than towards the isoxazole, confirming that the *cis*/*trans* preference is driven by the steric hindrance of the 3,5 substituents of the ring. Therefore, also the configuration of the NO group and the planarity of the entire molecule seem to play a crucial role in modulating the activity of the described compounds.

Conclusion

In conclusion, we have synthesized and evaluated in vitro the antimicrobial activity of a new class of 4-nitrosopyrazoles. Compound **6c** exhibits an antifungal activity 9-fold more potent than miconazole and is 20-fold more selective against *C. neoformans*. **6c** was also 8- and 125-fold more potent than amphotericin B and fluconazole, respectively. Compounds **6b–e** were also active against *Candida* spp., *A. fumigatus* and *T. mentagrophytes* at concentrations comparable with those of miconazole. We should point out that all compounds belonging both to the series **III** and **IV**, except **6a**, were active against *C. neoformans* at concentrations comparable with or smaller than fluconazole and amphotericin B. According to these in vitro results, compound **6c** could be a promising candidate for future development.

Experimental

Chemistry

All melting points were taken on a Buchi-Tottoli capillary apparatus and are uncorrected. IR spectra were determined in bromoform with a Jasco FT/IR 5300 spectrophotometer. ^{1}H and ^{13}C NMR spectra were measured at 200 and 50.3 MHz, respectively, in $(CD_3)_2SO$ solution unless otherwise specified, using a Bruker AC series 200 MHz spectrometer (TMS as internal reference). Mass spectra were obtained with an HP 5889A-GC/MS apparatus. Column chromatography was perfomed with Merck silica gel 230–400 Mesh ASTM. Analyses indicated by the symbols of the elements or functions were within ±0.4% of theoretical values.

1-(5-Methyl-3-isoxazolyl)-1,3-butanedione **1** and 1-(5-methyl-3-isoxazolyl)-2-hydroxyimino-1,3-butanedione **2** were prepared according to the procedures previously reported.[2] Derivatives **4** and **5b,c** were prepared according to ref 17.

General methods. For the preparation of 3-(3-methyl-4-nitroso-1-*R*-1*H*-5-pyrazolyl)-5-methylisoxazoles **6a**–**g**, 3-(5-methyl-4-nitroso-1-*R*-1*H*-3-pyrazolyl)-5-methylisoxazoles **7a**–**f**, 3-(3-methyl-1-*R*-1*H*-5-pyrazolyl)-5-methylisoxazole **4d**, and 3-(5-methyl-1-*R*-1*H*-3-pyrazolyl)-5-methylisoxazole **5d**.

Method A. To an aqueous suspension of finely powdered hydroxyimine **2** 0.97 g (5 mmol), a stoichiometric amount of suitable hydrazine salt dissolved in distilled water (20 mL) was added dropwise at room temperature. The reaction mixture was stirred until no starting material was detected (TLC). After completion of reaction (1–24 h), the mixture was extracted with dichloromethane (3×100 mL) and dried (anhydrous sodium sulphate). Removal of the solvent under reduced pressure gave a blue-green residue, which was purified by column chromatography.

Method B. A mixture of 1,3-diketone **2**, 0.97 g (5 mmol), and suitable equimolar hydrazine salt, in 20 mL of dry dichloromethane, was stirred at room temperature until starting material disappeared. After completion of the reaction the solvent was evaporated under reduced pressure and the residue chromatographed by silica gel column.

Method C. The equimolar mixture of diketone **2** and suitable hydrazine salt (5 mmol) in dry ethanol (30 mL) was stirred at 0 °C to room temperature for 72 h. At the end of the reaction, the solvent was removed and the residue chromatographed as above.

Method D. As method C but the reactants were refluxed for 1 h. Removal of the solvent afforded a dark residue which was purified by silica gel column.

Method E. As method A, except for the 1,3-diketone **2** that was dissolved in water with pellets of sodium hydroxide.

Method F. The equimolar mixture of **2** and **3g** (5 mmol) in AcOH (30 mL) was refluxed for 1 h and then poured in ice–water. After neutralization with sodium hydrogen carbonate, extraction with dichloromethane and removal of the solvent left a residue which was chromatographed.

Yields, melting points, recrystallization solvent, eluants of column chromatography, molecular ion (*m*/*z*), molecular formula, IR and ^{1}H NMR spectral data are reported in Tables 1 and 2, and ^{13}C NMR in Table 3.

3-(1,3-Dimethyl-4-nitro-1*H*-3-pyrazolyl)-5-methyl-4-nitroisoxazole (8b).[17] Eluted with petroleum ether (bp 40–60 °C):ethyl acetate 1:1, yield 90%, and recrystallized from ethanol, mp 124 °C. ^{1}H NMR (DMSO-d_6) δ 2.55 (3H, s, CH_3), 2.98 (3H, s, CH_3), 3.87 (3H, s, CH_3); ^{13}C NMR (DMSO-d_6) δ 13.14 (q), 13.64 (q), 38.42 (q), 127.80 (s), 130.08 (s), 132.49 (s), 145.62 (s), 147.51 (s), 174.12 (s); *m*/*z* 267. Anal. $C_9H_9N_5O_5$ (C,H,N).

3-(1-Ethyl-3-methyl-4-nitro-1*H*-5-pyrazolyl)-5-methylisoxazole (9c). To a cooled solution (−5 °C) of nitric acid 65% (2 mL), 2 mmol of **6c** were added in small portions. The reaction mixture was stirred for 5 h maintaining the temperature at 5 °C. After disappearance of the starting material (TLC), the solution was poured in ice–water and neutralized with sodium carbonate. The white solid formed was filtered, air dried, and purified by crystallization.

3-(4-Amino-1-ethyl-3-methyl-1*H*-5-pyrazolyl)-5-methylisoxazole (10c) and 3-(4-amino-1-ethyl-5-methyl-1*H*-3-pyrazolyl)-5-methylisoxazole (11c). Compound **6c** or **7c** (2 mmol) was slowly added into a stirred and cooled (0 °C) solution of $SnCl_2$ (2 g) in 2 mL of hydrochloric acid (37%). Within 10 min, the reaction was complete (TLC). Neutralization with sodium carbonate and extraction with dichloromethane gave an oil, which was purified by column chromatography.

Lipophilicity measurements. The relative lipophilicity of the compounds was measured by reversed-phase TLC according to the method already described.[22] R_M values were calculated from the experimental R_f values (calculated as mean values for five determinations) according to the equation $R_M = \log[(1/R_f)-1]$. Higher R_M values correspond to higher lipophilicity.

Crystal X-ray diffraction analysis. Single crystal X-ray diffraction analyses were carried out at room temperature by a Siemens AED diffractometer for **6e** and an Enraf–Nonius CAD4 diffractometer for **7e**. In both cases graphite-monochromated CuK_α radiation was used ($\lambda = 1.54138$ Å). Relevant details concerning data collection and structure refinements were provided to the editor. For **6e**, crystal decay was observed, and the data were rescaled accordingly. The intensity data were processed with a peak-profile analysis procedure and corrected for Lorentz and polarization effects. The phase problem was solved by direct methods using SIR97.[18] Full matrix least-squares refinements were carried out by SHELXL97[19] on F^2, using all measured unique data. Anisotropic thermal displacement parameters were refined for all non-hydrogen atoms. H atoms

were introduced in idealized positions, riding on their carrier atoms, for **6e** and for the methyl groups of **7e**. The remaining H atoms of **7e** were located on ΔF maps and refined isotropically. Programs PARST97[20] and ZORTEP[21] were used for analyzing and drawing the molecular structures. Use was made of the packages of the Cambridge Structural Database (release October 1998). All the calculations were performed on a Digital Alpha 255 workstation at the Centro di Studio per la Strutturistica Diffrattometrica del C.N.R. in Parma.

Biological assays

Compounds. Test compounds were dissolved in DMSO at an initial concentration of 200 μM and then were serially diluted in culture medium.

Cells. Cell lines were from American Type Culture Collection (ATCC); bacterial and fungal strains were either clinical isolates (obtained from Clinica Dermosilopatica, University of Cagliari) or collection strains from ATCC. H9/III_B, MT-4 and C8166 cells [grown in RPMI 1640 containing 10% fetal calf serum (FCS), 100 UI/mL penicillin G and 100 μg/mL streptomycin] were used for anti-HIV-1 assays. Cell cultures were checked periodically for the absence of mycoplasma contamination with a MycoTect Kit (Gibco).

Virus. Human immunodeficiency virus type-1 (HIV-1, III_B strain) was obtained from supernatants of persistently infected H9/III_B cells. HIV-1 stock solution had a titer of 5×10^7 cell culture infectious dose 50 ($CCID_{50}$)/mL.

Antiviral assays. Activity against the HIV-1 multiplication in acutely infected cells was based on inhibition of virus-induced cytopathogenicity in MT-4 cells.[23] Briefly, 50 μL of RPMI/10% FCS containing 1×10^4 cells were added to each well flat-bottomed microtiter trays containing 50 μL of medium and serial dilutions of test compounds. 20 μL of an HIV-1 suspension containing 100 $CCID_{50}$ were then added. After a 4 day incubation at 37 °C, the number of viable cells was determined by the 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl-tetrazolium bromide (MTT) method.[24,25] Cytotoxicity of compounds, based on the viability of mock-infected cells as monitored by the MTT method, was evaluated in parallel with their antiviral activity.

Antibacterial assays. *Staphylococcus aureus*, group D *Streptococcus*, *Shigella* and *Salmonella* spp. were recent clinical isolates. Assays were carried out in nutrient broth (DIFCO), pH 7.2, with an inoculum of 10^3 bacterial cells/tube. Minimum inhibitory concentrations (MIC) were determined after incubation at 37 °C for 18 h in the presence of serial dilutions of test compounds.

Antimycotical assays. Yeast inocula were obtained by properly diluting cultures incubated at 37 °C for 30 h in Sabouraud dextrose broth to obtain 5×10^3 cells/mL. On the contrary, dermatophyte inocula were obtained from cultures grown at 37 °C for 5 days in Sabouraud dextrose broth by finely dispersing clumps with a glass homogenizer and then diluting to 0.05 OD_{590}/mL. Then, 20 μL of the above suspensions were added to each well of flat-bottomed microtiter trays containing 80 μL of medium with serial dilutions of test compounds, and were incubated at 37 °C. Growth controls were visually determined after 2 days (yeast) or 3 days (dermatophytes). MIC was defined as the compound concentration at which no macroscopic sign of fungal growth was detected. The minimal germicidal concentrations (MBC or MFC) were determined by subcultivating in Sabouraud dextrose agar samples from cultures with no apparent growth.

Linear regression analysis. Viral and cell growth at each drug concentration was expressed as percentage of untreated controls and the concentrations resulting in 50% (EC_{50}, CC_{50}) growth inhibition were determined by linear regression analysis.

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