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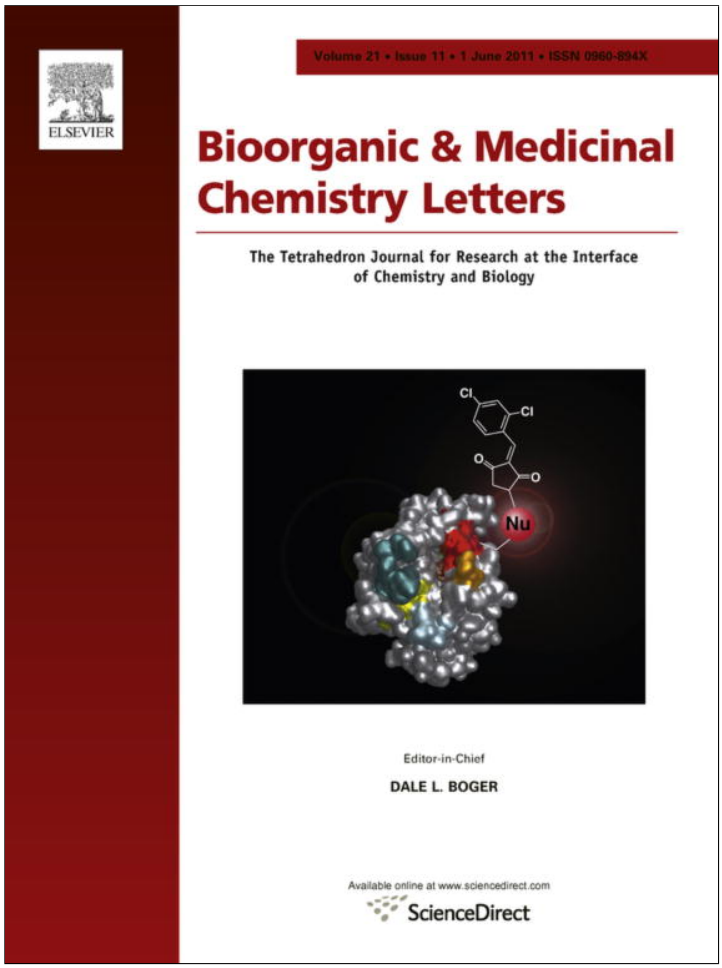

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Synthesis and in vitro antimicrobial activities of new (cyano-NNO-azoxy) pyrazole derivatives

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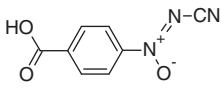
ABSTRACT

The antibacterial and antifungal activity of a series of products, in which the 1,5-dimethyl-4-(cyano-NNO-azoxy)pyrazol-3-yl and 1,3-dimethyl-4-(cyano-NNO-azoxy)pyrazol-5-yl moieties were linked to pyridine, pyrazole, isoxazole, thiophene and the furan ring, were examined. No molecule displayed activity against the Gram-negative bacteria tested. Conversely, some compounds displayed activity against two *Staphylococcus* aureus strains, including the methicillin resistant strain. All compounds displayed interesting antifungal activity, the most active compound of the series being the thiophene derivative **7a**. This compound's activity against *Candida krusei* and *Candida glabrata* (MIC = 0.25 and 0.5 μg/mL, respectively), two fungal species resistant to azoles, is noteworthy. The presence of the cyano function appeared essential for activity.

The azoxycyanide functional group (–N(O)=N–CN) was originally discovered in the antibiotic calvatic acid (4-carboxyphenyl-ONNazoxycyanide, 4-(cyano-NNO-azoxy)benzoic acid), which was first isolated from a culture broth of *Calvatia lilacina* (BERK.) Henn. P.,[1] then from cultures of *Calvatia craniformis* (SHW.) Fr.,[2] and *Lycoperdon pyriforme*.[3]



Calvatic acid

Calvatic acid displays significant antifungal and antibacterial activity, as well as in vitro and in vivo antitumor activity.[2,4] It also has activity against a number of strains of Helicobacter pylori, including metronidazole resistent ones.[5] The antibiotic also shows a range of specific biological functions; among these are inhibition of [^{3}H] colchicine binding to soluble tubulin, inhibition of human placenta glutathione transferase, as well as of ornithine decarboxylase.[6–8] The structure of Calvatic acid has been used as a starting point in several other studies; in particular, the azoxycyanide function has been inserted in a variety of aryl and heteroaryl vectors.[9–13] The resulting compounds displayed the general activities of the lead, but with a range of potency and specificity. They are superior antifungal agents compared to the parent compound. These derivatives have also been examined as potential agricultural fungicides.[14] The results are reported of a study of the antibacterial and antifungal activity of a series of products in which the 1,5-dimethyl-4-(cyano-NNO-azoxy)pyrazol-3-yl and 1,3-dimethyl-4-(cyano-NNO-azoxy)pyrazol-5-yl moieties R^3 and R^5 (Table 1) were linked to pyridine, pyrazole, isoxazole, thiophene and furan ring. In order to elucidate the significance of the cyano function on activity, the carbamoyl **10** and tosyl **11** analogues of **7a**, the most active compound of the series, were also examined.

Scheme 1 outlines the preparation of the final azoxycyano derivatives. The starting materials were the 1-methyl-4-nitrosopyrazoles derivatives **1**–**9**, bearing methyl and heteroaryl substituents at 3- and 5- positions. These products were transformed into the final compounds by regiospecific synthesis, as described by Fruttero et al.[15] and later modified by Wood et al,[13] with the exception of the oxidative-breakdown-susceptible furan derivative **9a**, for which the procedure was partially modified. Nitrosoderivatives were treated in dry CH_2Cl_2 with a mixture of cyanamide (NH_2CN) and (diacetoxy)iodobenzene (IBA), giving the expected final products, probably through the intermediate formation of cyanonitrene. All products showed the fragment ions $[M-40]^+$in their mass spectra, due to loss of CN_2, typical of this class of compounds.[16] Compound **10** was easily obtained by bubbling HCl through a THF/H_2O solution of **7a**, following the general procedure described elsewhere,[12] while compound **11** was synthesized by action of the nitrene precursor *N*-tosyliminoiodinane, prepared by a known procedure,[17] on **7** dissolved in acetonitrile containing activated 4 Å molecular sieves (MS) and CuCl (Scheme 2).

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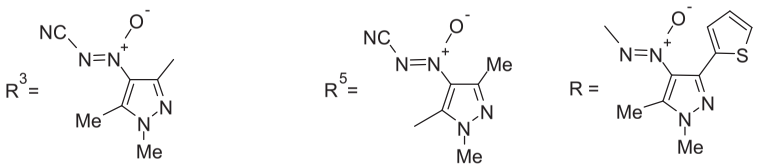
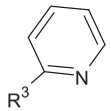
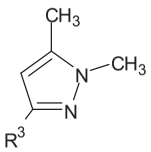
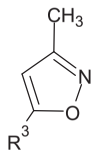
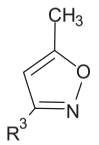
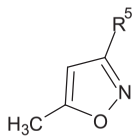
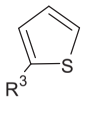
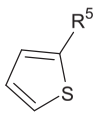
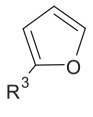
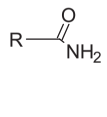
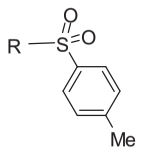
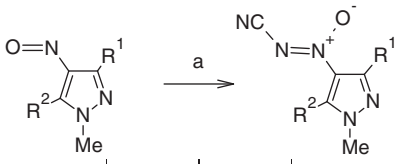
Table 1
In vitro activities of the designed compounds (MIC, μg/mL)



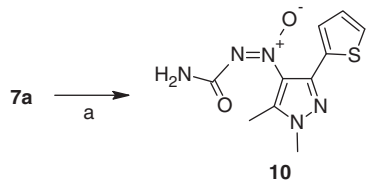
Species	1a	2a	3a	4a	5a	6a	7a	8a	9a	10	11
											
S. aureus MR	>128	32	32	2	8	>128	4	ND	ND	ND	ND
S. aureus MS	>128	64	32	4	16	>128	8	ND	ND	ND	ND
P. mirailis	>128	>128	>128	>128	>128	>128	>128	ND	ND	ND	ND
E. coli	>128	>128	>128	>128	>128	>128	>128	ND	ND	ND	ND
P. aeruginosa 1	>128	>128	>128	>128	>128	>128	>128	ND	ND	ND	ND
P. aeruginosa 2	>128	>128	>128	>128	>128	>128	>128	ND	ND	ND	ND
C. albicans 31	32	64	64	128	128	32	1	4	2	>128	>128
C. albicans 41	32	16	>128	64	128	32	0.5	ND	ND	ND	ND
C. albicans 47	32	128	>128	128	>128	128	1	4	4	>128	>128
C. albicans 48	32	128	>128	128	>128	128	1	4	4	>128	>128
C. kruse 31	32	16	>128	64	128	32	0.5	1	1	>128	>128
C. krusei 43	32	16	>128	32	64	32	1	1	2	>128	>128
C. krusei 48	32	8	>128	64	128	32	0.5	2	2	>128	>128
C. tropicalis 33	64	128	>128	128	>128	>128	8	32	128	>128	>128
C. tropicalis 11	128	128	>128	128	>128	128	8	32	32	>128	>128
C. tropicalis 15	ND	ND	ND	ND	ND	ND	ND	64	128	>128	>128
C. tropicalis 22	64	128	>128	>128	>128	>128	16	32	128	>128	>128
C. glabrata 30	64	64	>128	128	128	128	0.25	2	1	>128	>128
C. glabrata 32	16	4	>128	32	64	16	0.25	2	2	>128	>128
C. glabrata 46	64	64	>128	128	128	128	0.25	2	1	>128	>128
C. glabrata 49	64	128	>128	128	128	64	0.25	2	1	>128	>128
C. parapsilosis 26	64	128	>128	128	128	128	8	16	8	>128	>128
C. parapsilosis 39	ND	ND	ND	ND	ND	ND	ND	16	16	>128	>128
C. parapsilosis 44	64	128	>128	128	128	128	8	ND	ND	ND	ND
C. parapsilosis 19	64	128	>128	128	128	>128	16	32	64	>128	>128
Cr. neoformans 14	32	16	16	2	4	8	2	0.25	0.25	64	64
Cr. neoformans 27	32	16	16	2	4	8	2	0.25	0.25	64	64
Cr. neoformans 30	32	8	16	2	8	8	2	0.5	0.25	128	128
Cr. neoformans 25	32	8	16	2	8	8	2	0.5	0.25	128	128
C. albicans ATCC	ND	ND	ND	ND	ND	ND	ND	4	1	>128	>128
C. krusei ATCC	16	16	>128	32	64	16	0.5	1	1	>128	>128
C. parapsilosis ATCC	32	128	>128	128	128	>128	16	32	32	>128	>128

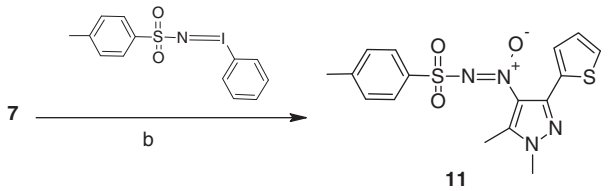
ND Not done.



Nitroso derivatives	R^1	R^2	Azoxy cyanides
1	Me	Me	1a
2		Me	2a
3		Me	3a
4		Me	4a
5		Me	5a
6	Me		6a
7		Me	7a[18]
8	Me		8a[19]
9		Me	9a[20]

Scheme 1. Reagents and conditions: (a) NH_2CNNH_2CN, (diacetoxy)iodobenzene (IBA), dry CH_2Cl_2, 30 °C.



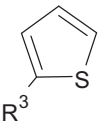
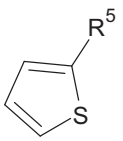
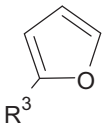


Scheme 2. Reagents and conditions: (a) HCl gas, THF/H_2O, 0 °C. (b) *N*-tosyliminoiodinane, CuCl, 4 Å MS, dry acetonitrile, rt.

The first seven products were tested against two strains of *Staphylococcus aureus*, one resistant (MR) and the other susceptible (MS) to methicillin, three species of Gram-negative bacteria, *Proteus mirabilis, Escherichia coli* and *Pseudomonas aeruginosa,* six fungal species belonging to *Candida* spp., and *Cryptococcus neoformans* var. *neoformans.* Because both yeasts and *S. aureus* can be normal microbiota of human mucosa and skin, the yeasts and the two *S. aureus* isolates used in this study were collected from human sterile clinical specimens (blood and cerebrospinal fluid), Gram-negative bacteria were isolated from urine having a colony forming unit (CFU) count above the cutoff of $>10^5$ CFU. Antimicrobial assays were performed with a microdilution broth method, following standard protocols for in vitro antibacterial and antifungal susceptibility testing.[21,22] The results are in Table 1[23].

The antimicrobial data showed that none of the molecules displayed activity against the Gram negative bacteria tested. Conversely, except for **1a** and **6a**, they displayed some activity against both *S. aureus* strains, including the MR strain, against

Table 2
Comparison of in vitro activities of the designed compounds with that of the widely used systemic antifungal drugs (MIC, μg/mL)

Species	**7a**	**8a**	**9a**	Fluconazole	Voriconazole	Posaconazole	Amphotericinb
							
C. albicans 31	1	4	2	0.25	<0.008	0.008	0.5
C. albicans 41	0.5	ND	ND	0.12	<0.008	0.016	0.5
C. albicans 47	1	4	4	0.25	0.008	0.016	1
C. albicans 48	1	4	4	0.12	<0.008	0.008	0.5
C. krusei 31	0.5	1	1	32	0.12	0.25	0.5
C. krusei 43	1	1	2	64	0.5	0.5	1
C. krusei 48	0.5	2	2	32	0.25	0.25	1
C. tropicalis 33	8	32	128	1	<0.008	0.12	1
C. tropicalis 11	8	32	32	1	0.12	0.12	1
C. tropicalis 15	ND	64	128	1	0.06	0.25	1
C. tropicalis 22	16	32	128	2	0.06	0.25	0.5
C. glabrata 30	0.25	2	1	8	0.25	0.5	1
C. glabrata 32	0.25	2	2	>256	4	>8	0.5
C. glabrata 46	0.25	2	1	16	0.25	1	1
C. glabrata 49	0.25	2	1	8	0.12	0.25	0.5
C. parapsilosis 26	8	16	8	1	<0.008	0.03	0.5
C. parapsilosis 39	ND	16	16	1	0.016	0.06	0.5
C. parapsilosis 44	8	ND	ND	2	0.016	0.03	0.5
C. parapsilosis 19	16	32	64	1	0.008	0.03	0.5
Cr. neoformans 14	2	0.25	0.25	0.5	<0.008	0.03	0.25
Cr. neoformans 27	2	0.25	0.25	2	0.016	0.06	0.12
Cr. neoformans 30	2	0.5	0.25	4	0.016	0.12	0.5
Cr. neoformans 25	2	0.5	0.25	2	0.016	0.06	0.5
C. albicans ATCC	ND	4	1	0.12	0.008	0.008	0.5
C. krusei ATCC	0.5	1	1	32	0.25	0.25	1
C. parapsilosis ATCC	16	32	32	2	0.06	0.12	1

which erythromycin and ciprofloxacin are also inactive, as determined following the established clinical breakpoint $\geqslant 1$ μg/mL.

From this standpoint, the lowest MIC values were displayed by derivatives **4a**, **5a**, and **7a**, respectively bearing the 3-methyl-5-oxazolyl, 5-methyl-3-oxazolyl, and thiophen-2-yl substituent at the 3-position of the pyrazolylazoxycyanide scaffold R^3. As far as antifungal activity is concerned, the most active product was **7a**: this is a potent antifungal product active against almost all the species tested. *Candida parapsilosis* and *Candida tropicalis* isolates had higher MIC values than those of the other yeasts tested. The activity against *Candida krusei*, which displays intrinsic fluconazole resistance, and against *Candida glabrata*, which has acquired resistance to azoles, are of note (Table 2).[24] When the thienyl substituent was moved to position 5 of the pyrazole ring, to give compound **8a**, the level of activity remained high, MIC values being within $\pm 2 \log_2$ dilutions. Compound **8a** was particularly active against the strains of *Cryptococcus neoformans*, which was susceptible to all the pyrazolylazoxycyanide derivatives tested. When in **7a** the furan moiety was substituted for the thiophene, giving compound **9a**, activity against all the species under study remained high, declining only for the strains of *C. tropicalis*. The presence of the azoxycyano function in these products appeared to be essential to their activity: the cyano group was substituted in **7a** with two other electron-withdrawing moieties, the carbamoyl and the tosyl moiety respectively, giving products **10** and **11**, which were inactive.

In conclusion, interesting pyrazole derivatives displaying antifungal activity were developed. In particular, compounds **7a**, **8a**, **9a** deserve further structural modulation, owing to their potent action against *C. krusei* and *C. glabrata*, two fungal species resistant to azoles.

Supplementary data

Supplementary data (synthesis of intermediates **7**, **8**, full experimental procedures, physicochemical characterization, and elemental analyses for the compounds described, is available free of charge via the Internet at http://) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.03.101.

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18. *4-(Cyano-NNO-azoxy)-1,5-dimethyl-3-(thiophen-2-yl)-1H-pyrazole* (**7a**): FC (petroleum ether/EtOAc 7:3) gives **7a** (85%) as a brown solid. Mp 131–132 °C (EtOAc/hexane). IR (KBr DRIFT/cm^{-1}): 2195 (C≡N), 1460, 1326 (N(O)═N). ^{1}H NMR ($CDCl_3$, 300 MHz) δ (ppm): 7.72 (dd, ^3J = 3.7 Hz, ^4J = 1.2 Hz 1H, Th), 7.43 (dd, ^3J = 5.1 Hz, ^4J = 1.2 Hz, 1H, Th), 7.11 (dd, ^3J = 5.1 Hz and 3.7 Hz, 1H, Th), 3.90 (s, 3H, 1-CH_3-Pz), 2.66 (s, 3H, 5-CH_3-Pz). ^{13}C NMR ($CDCl_3$, 75 MHz) δ (ppm): 141.7, 140.7, 131.3, 129.8, 128.1, 127.5, 126.4, 110.8, 37.6, 12.6. ES-MS (70 eV, *m*/*z*): 247 (M^+, 100%) 207 (M−40). Anal. Calcd for $C_{10}H_9N_5OS$: C, 48.57; H, 3.67; N, 28.32. Found: C, 48.54; H, 3.71; N, 28.14.
19. *4-(Cyano-NNO-azoxy)-1,3-dimethyl-5-(thiophen-2-yl)-1H-pyrazole* (**8a**): general procedure was modified as follows: acetonitrile as reaction solvent, 50 °C reaction temperature, 1:1 nitroso/IBA molar ratio. FC (hexane/EtOAc 75:25) gives **8a** (30%) as a brown solid. Mp 92–93 °C dec. (EtOAc/hexane). IR (KBr DRIFT/cm^{-1}): 2185 (C≡N), 1458, 1367 (N(O)═N). ^{1}H NMR ($CDCl_3$, 300 MHz) δ (ppm): 7.63 (d, *J* = 4.8 Hz, 1H, Th), 7.21–7.18 (m, 2H, 2 Th), 3.75 (s, 3H, 1-CH_3-Pz), 2.55 (s, 3H, 3-CH_3-Pz). ^{13}C NMR ($CDCl_3$, 75 MHz) δ (ppm): 146.0, 136.1, 131.5, 130.3, 128.5, 127.6, 125.4, 110.8, 38.1, 14.7. ES-MS (70 eV, *m*/*z*): 247 (M^+) 207 (M−40, 100%). Anal. Calcd for $C_{10}H_9N_5OS$: C, 48.57; H, 3.67; N, 28.32. Found: C, 48.48; H, 3.59; N, 28.28.
20. *4-(Cyano-NNO-azoxy)-3-(furan-2-yl)-1,5-dimethyl-1H-pyrazole* (**9a**): A mixture of the nitroso-derivative **9** (0.57 g, 3 mmol) and cyanamide (0.15 g, 3.6 mmol) in methylene chloride (5 mL) was treated at 0 °C with (diacetoxyiodo)benzene (0.97 g, 3 mmol) in portions over 15 min. The reaction mixture was directly deposited into the column and purified by FC (CH_2Cl_2/acetone 99.75:0.25) to obtain **9a** (0.10 g, 14%) as a yellow solid. Mp 152–154 °C dec. (EtOH). IR (KBr DRIFT/cm^{-1}): 2188 (C≡N), 1453, 1344 (N(O)═N). ^{1}H NMR ($CDCl_3$, 300 MHz) δ (ppm): 7.56 (d, ^3J = 1.5 Hz, 1H, Fu), 7.22 (d, ^3J = 3.6 Hz, 1H, Fu), 6.54 (dd, ^3J = 3.6 Hz and 1.5 Hz, 1H, Fu), 3.95 (s, 3H, 1-CH_3-Pz), 2.68 (s, 3H, 5-CH_3-Pz). ^{13}C NMR ($CDCl_3$, 75 MHz) δ (ppm): 144.05, 143.98, 140.5, 138.4, 126.1, 114.2, 111.7, 110.8, 37.9, 12.7. ES-MS (70 eV, *m*/*z*): 231 (M^+,100%), 191 (M−40,). Anal. Calcd for $C_{10}H_9N_5O_2$: C, 51.95; H, 3.93; N, 30.29. Found: C, 51.93; H, 3.75; N, 30.18.
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23. *In vitro activity*. The yeast isolates used in this study were collected from human sterile clinical specimens (blood and cerebrospinal fluid) to be sure of their pathogenic role. After overnight growth on Sabouraud dextrose agar at 35 °C, each yeast isolate was suspended in 5 mL of sterile distilled water and thoroughly vortexed to achieve a smooth suspension. Turbidity (read at a wavelength of 530 nm) was adjusted to a McFarland standard of 0.5 with water. This suspension (approximately $1–5 \times 10^6$ CFU/mL) was used as inoculum for susceptibility testing. Antifungal susceptibility testing was performed with a microdilution broth method using 96-well microtiter plates. The wells of each row contained a single compound dissolved in DMSO and diluted in RPMI 1640 medium buffered with MOPS 0.165 M and supplemented with 2% glucose. Ten wells in the row contained ten different scalar concentrations of the compound, ranging from 0.25 mg/L to 256 mg/L. For each isolate, the inoculum suspension was diluted twice with RPMI 1640 medium (1:100 and then 1:20). Aliquots (0.1 mL) of the latter dilution were then placed in 11 wells of a single row (10 wells contained the drug, the 11th as growth control, the 12th as blank). The plates were incubated at 35 °C. An initial visual examination was made after 24 h of incubation, and the lowest concentration that had inhibited visible growth was recorded as the MIC. After 48 h of incubation, the panels were analyzed spectrophotometrically (after shaking), and the MIC was recorded as the concentration that produced a 50% reduction in turbidity compared with that of the growth-control well. The 48 h readings were used to analyze results. Three quality control strains were included: *C. krusei* ATCC® 6258, *C. parapsilosis* ATCC® 22019, *Candida albicans* ATCC® 90028. Antibacterial susceptibility was tested as for yeasts, with a microdilution broth method using 96-well microtiter plates; Mueller Hinton broth was used instead of RPMI 1640 as medium. After overnight growth on Mueller Hinton broth at 35 °C, each bacterial isolate was diluted to achieve a suspension of approximately $1–5 \times 10^8$ CFU/mL. Aliquots (0.1 mL) of the latter dilution was then placed in 11 wells of a single row and incubated at 35 °C. Reading was after 24 h of incubation, and the lowest concentration that had inhibited visible growth was recorded as the MIC.
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