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Short communication

Synthesis, anti-mycobacterial, anti-trichomonas and anti-candida in vitro activities of 2-substituted-6,7-difluoro-3-methylquinoxaline 1,4-dioxides

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Abstract

A new series of 23 6,7-difluoro-3-methyl-2-phenylthio/phenylsulfonyl/phenylsulfinyl/benzylamino/phenylamino-quinoxaline 1,4-dioxides variously substituted in the phenyl moiety, was synthesized and submitted to in vitro evaluation for anti-mycobacterial, anti-trichomonas, anti-candida, anti-mycoplasma and antibacterial activities. In anti-mycobacterial assays, several compounds resulted active (MIC_{90} = 2.0–4.0 μg/ml) against *Mycobacterium tuberculosis* H37Rv. Anti-trichomonas screening showed a generally good activity of all compounds (MBC = 0.39–25.0 μg/ml) versus *Trichomonas vaginalis*, in particular the derivatives **5a,d, 7a, 9** and **11c** ranged 0.39–0.78 μg/ml (metronidazole MBC = 12.5 μg/ml). Results of anti-candida assays showed that derivatives **7a, 8a,d** and **9** were active against several species of *Candida* (*C. albicans, C. krusei, C. parapsilosis* and *C. glabrata*), having MIC_{50} between 3.9 and 31.25 μg/ml. The latter compounds were also submitted to anti-mycoplasma assay against *Mycoplasma hominis*, the results obtained showed that **7a, 8a,d** and **9** inhibited the growth of the mycoplasma at the concentration of 0.1 mg/ml. In antibacterial tests only a few compounds showed an MIC_{50} lower than 62.5 μg/ml against representative strains of Gram-positive and Gram-negative bacteria (*Escherichia coli, Klebsiella pneumoniae, Staphylococcus aureus, Vibrio alginolyticus* and *Pseudomonas aeruginosa*).

Keywords: Quinoxaline 1,4-dioxides; Anti-mycobacterial; Anti-trichomonas; Anti-candida activity; SAR

1. Introduction

In the last two decades, an increasing interest on the biological properties of the quinoxaline 1,4-dioxides has been reported. Antibacterial activity [1–5], animal growth promoting in feed additives [6–8], hypoxia-selective activity [9], genotoxicity against *Escherichia coli* and *S. typhimurium* [10] are well documented. In this context we recently reported the anti-mycobacterial and anti-candida activities of the compounds of Fig. 1 which confirm the potentiality of this nucleus.

In particular, we could observe that the 3-methyl-2-phenylthioquinoxaline 1,4-dioxides bearing alternatively a chlorine atom or a trifluoromethyl group at C-6 or C-7 as well as two fluorine atoms in C-6 and C-7 were endowed with good anti-mycobacterial activity (MIC_{90} between 0.39 and 0.78 μg/ml) [11] within the TAACF screening program [12], while a general good anti-candida activity (*Candida albicans, Candida glabrata, Candida krusei* and *Candida parapsilosis*) was recorded for all terms of the series [11].

Analogous anti-mycobacterial activities were obtained recently by Zarranz et al. [13] for a new series of 3-methylquinoxaline 1,4-dioxides.

Our encouraging results as well as the reports on the literature prompted us to extend the previous series of 6,7-difluoroquinoxaline 1,4-dioxides in order to study structure–activity relationships. Thus, we have designed the compounds of Schemes 1 and 2 where at position 3 of the heterocycle is always present a methyl group for synthetic reasons, while at position 2 we placed variously mono-, di- or tri-substituted phenyl ring linked by thio, sulfonyl or sulfinyl bridge. In addition we have examined the substitution with anilino or benzylamino group.

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R = Cl, S-Ph, SO_2Ph;
R_1 = H, Br;
R_2/R_3 = H, Cl, F, CF_3, CH_3, O-Et;

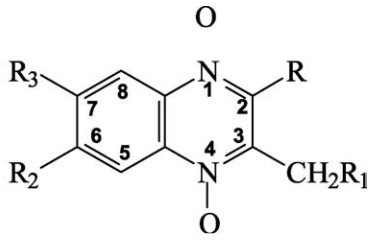


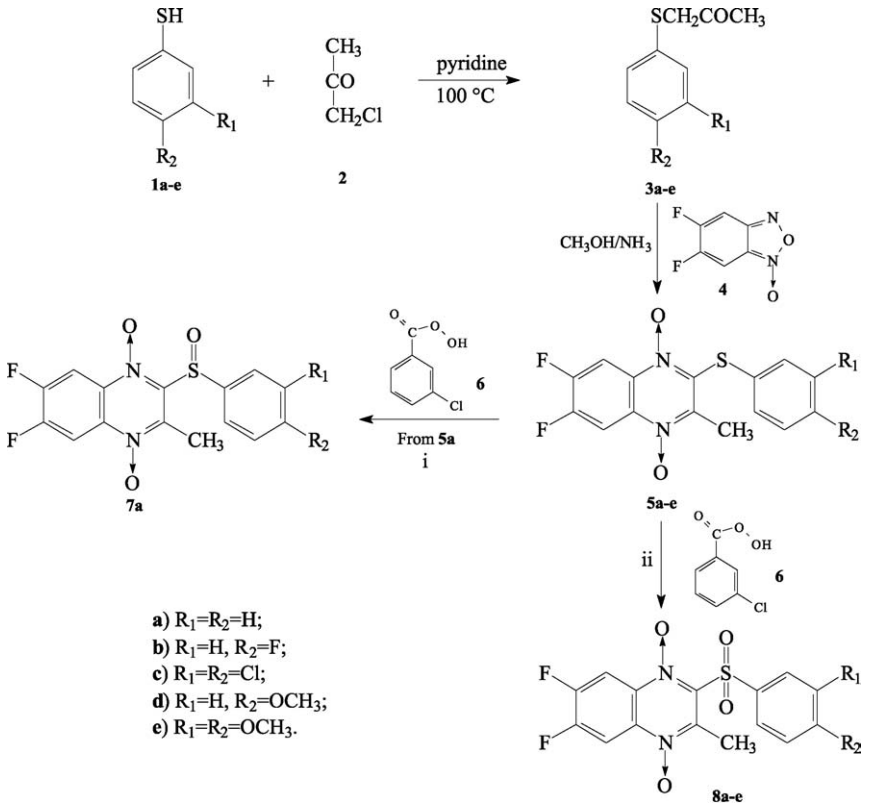
Fig. 1. Quinoxaline 1,4-dioxides previously described.

2. Chemistry

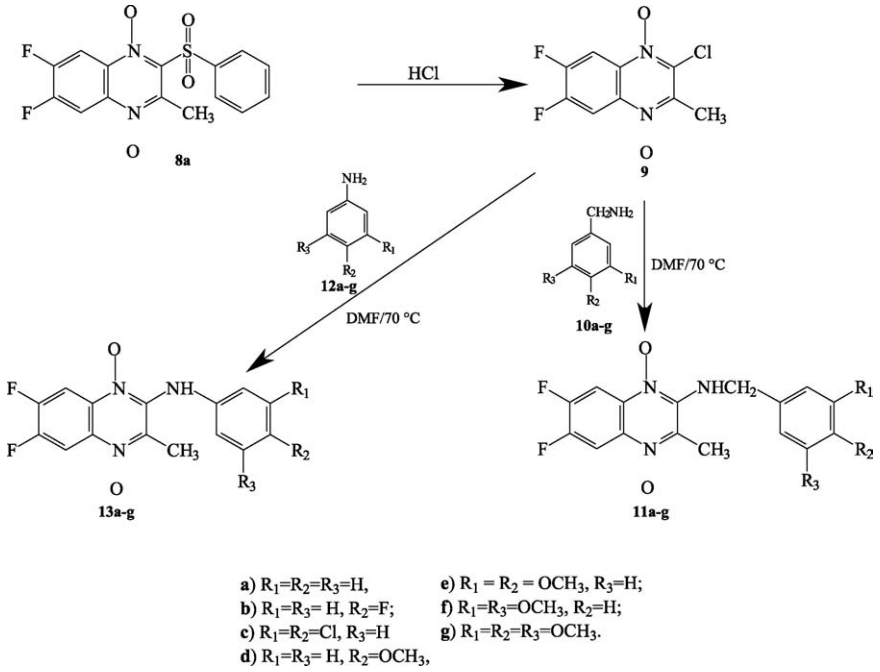
The preparation of 6,7-difluoro-3-methyl-2-penylthio (**5a–e**), -2-phenylsulfinyl (**7a**) and -2-phenylsulfonyl (**8a–e**) quinoxalines is reported in Scheme 1. The intermediate acetonylphenyl sulfides (**3a–e**) were obtained by reaction of thiophenol derivatives (**1a–e**) with chloroacetone (**2**) in the presence of pyridine at 100 °C, adapting a general procedure reported by Banfield et al. [14] for the preparation of acetonylaryl sulfides. Successive nucleophilic attack by the known 5,6-difluorobenzofuroxane (**4**) [11] in methanol, made alkaline with saturated dry ammonia methanol solution, afforded the compounds (**5a–e**) in moderate yields. Oxidation of the sulfides with a slight excess (1:2.5 molar ratio) of *m*-chloroperbenzoic acid (MCPBA) in chloroform gave the 1,4-dioxides (**8a–e**) in high yields. Compound **8a** was previously described by us [11]. However, in this occasion, we have discovered that when **5a** underwent oxidation using 1:1.2 molar ratio with MCPBA it gave the sulfinylquinoxaline 1,4-dioxide (**7a**). For the preparation of the compounds **11a–g** and **13a–g**, we used the well-experimented Scheme 2 first converting the sulfide **8a** into the 2-chloro-3-methyl-6,7-difluoroquinoxaline 1,4-dioxide (**9**) [11] by treatment with concentrated hydrochloric acid solution. Compound **9** underwent nucleophilic displacement of chlorine by both benzylamino (**10a–g**) and anilino (**12a–g**) intermediates to give the desired compounds **11a–g** and **13a–g**, respectively, in good yields.

3. Microbiology

About the biological screening, all new compounds and some previous synthesized derivatives were submitted to anti-mycobacterial (*Mycobacterium tuberculosis* H37Rv), anti-candida (*C. albicans, C. glabrata, C. krusei* and *C. parapsilosis* clinical isolates) and antibacterial (Gram-positive: *Staphylococcus aureus* ATCC 2913 and Gram-



Scheme 1. Method of preparation of quinoxalines 6,7-difluoro-3-methyl-2-phenylthio/phenylsulfinyl/phenylsulfonyl 1,4-dioxides (**5a–e, 7a**, and **8a–e**). Conditions: (i) **5a** and MCPBA 1:1.2 molar ratio; (ii) **5a–e** and MCPBA 1:2.5 molar ratio.



Scheme 2. Method of preparation of quinoxalines 6,7-difluoro-3-methyl-2-benzylamino/phenylamino-quinoxaline 1,4-dioxides (**11a–g**, and **13a–g**).

negative: *E. coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 700603, *Pseudomonas aeruginosa* ATCC 27853 and the environmental isolate *Vibrio alginolyticus*) assays. Furthermore, in order to investigate this type of quinoxalin 1,4-dioxides against sexual transmitting diseases, the same compounds were also submitted to anti-trichomonas screening against *Trichomonas vaginalis*, and compounds which exhibited the best activity against several species of *Candida*, were also submitted to screening against *Mycoplasma hominis*.

4. Results and discussion

Quinoxaline 1,4-dioxides (**5a–e, 7a, 8a–e, 9, 11a–g** and **13a–g**) were evaluated in vitro for antibacterial (*S. aureus, E. coli, V. alginolyticus, K. pneumoniae* and *P. aeruginosa*), antifungal (*C. albicans, C. glabrata, C. krusei* and *C. parapsilosis*), anti-mycobacterial (*M. tuberculosis*), anti-trichomonas (*T. vaginalis*), and anti-mycoplasma (*M. hominis*) activities. The results obtained show that various compounds possess an interesting activity against several tested strains.

With regard to antibacterial activity, the obtained results mainly indicate that most of tested compounds exhibited low activity against Gram-positive and Gram-negative bacteria showing MIC values ranging from 62.5 to 500 μg/ml with a few exceptions. Compounds **5e, 7a** and **8a,d** (MIC = 31.25 μg/ml) and **9** (MIC = 15.6 μg/ml) were the most active against *E. coli*, while only compound **5e** shows MIC = 31.25 μg/ml against *S. aureus* and *K. pneumoniae*. This moderate activity confirms our previous report [11] that 3-methylquinoxaline 1,4-dioxides in general do not display significant activity versus the mentioned bacteria. However, the best activity was exhibited when a more or less oxidized sulfur atom connects the C-2 position of quinoxaline scaffold with the phenyl moiety as phenylthio (**5e**), phenylsulfinyl (**7a**) and phenylsulfonyl (**8a,d**) derivatives, or a chlorine atom is present in C-2 (**9**). About the phenyl moiety, the most favorable resulted none for **7a** and **8a**, a *para* methoxy group for **8d**, or both *meta* and *para* methoxy group for **5e**.

The in vitro anti-candida activity was first tested against clinically isolated strains of *C. albicans*, using miconazole as reference drug (MIC = 3.9 μg/ml), the MICs ranged between 7.8 and 250 μg/ml. Compounds that exhibited MCs ⩽ 15.6 μg/ml (**7a, 8a,d** and **9**) were submitted to screening against further strains of different Candida species (*C. glabrata, C. krusei* and *C. parapsilosis*). From this screening, the results reported in Table 1 show that although none of the tested compounds exhibited a better activity than miconazole, MIC and minimum fungicidal concentration (MFC) values ranged between 3.9 and 31.25 μg/ml. We must point out that also in this screening the best derivatives were those having phenylsulfinyl (**7a**), phenylsulfonyl (**8a,d**), or a chlorine atom (**9**) on C-2 position, and no substituent (**7a, 8a**) or a *para* methoxy group (**8d**) on the phenyl moiety. Furthermore, the same compounds (**7a, 8a,d** and **9**) were evaluated against *M. hominis*. In this case the results showed that the growth of this bacterium was inhibited at the concentration of 0.1 mg/ml.

In Table 2 we have reported the results of an in vitro anti-tubercular screening of 16 derivatives that showed a general good activity against *M. tuberculosis* (MCs ⩽ 4 μg/ml). In particular, the compounds **5b,d,e** and **9**, exhibited MIC = 2 μg/ml (in comparison with rifampin MIC = 1 μg/ml). Antitubercular assays seem to confirm our

Table 1
In vitro evaluation of anti-candida activity against clinical isolates *C. glabrata, C. krusei* and *C. parapsilosis* (MIC_{100} μg/ml) of compounds having MICs = 15.6 μg/ml against *C. albicans*

	7a		**8a**		**8d**		**9**		Miconazole
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC
C. albicans	7.8	7.8	7.8	15.6	15.6	15.6	7.8	15.6	3.9
C. glabrata	7.8	7.8	15.6	15.6	15.6	31.25	3.9	7.8	0.4
C. krusei	3.9	7.8	7.8	7.8	3.9	7.8	3.9	3.9	0.9
C. parapsilosis	15.6	31.25	31.25	31.25	31.25	31.25	15.6	31.25	0.4

Table 2
In vitro evaluation of compounds having MICs = 4 μg/ml against *M. tuberculosis* H37Rv

Compound	MIC (μg/ml)	Compound	MIC (μg/ml)	Compound	MIC (μg/ml)
5b	2	**8c**	4	**13a**	4
5c	4	**8d**	4	**13b**	4
5d	2	**9**	2	**13c**	4
5e	2	**11a**	4	**13d**	4
8a	4	**11b**	4		
8b	4	**11d**	4	Rifampin	1

Table 3
In vitro evaluation of anti-trichomonas activity against *T. vaginalis* (MIC_{100} μg/ml)

Compound	MIC				Compound	MIC			
	After 24 h	After 48 h	After 72 h	After 96 h		After 24 h	After 48 h	After 72 h	After 96 h
5a	3.12	0.78	0.39	0.39	**11c**	6.25	0.78	0.78	0.78
5b	>50	50	50	50	**11d**	>50	25	25	25
5c	12.5	3.12	3.12	3.12	**11e**	25	12.5	6.25	6.25
5d	6.25	0.78	0.78	0.78	**11f**	25	12.5	6.25	6.25
5e	>50	25	25	12.5	**11g**	25	12.5	6.25	6.25
7a	1.56	1.56	0.78	0.78	**13a**	>50	25	25	25
8a	1.56	1.56	1.56	1.56	**13b**	>50	12.5	3.12	3.12
8b	6.25	3.12	3.12	3.12	**13c**	>50	25	25	25
8c	6.25	3.12	3.12	3.12	**13d**	>50	25	12.5	12.5
8d	6.25	3.12	1.56	1.56	**13e**	>50	25	12.5	12.5
8e	3.12	3.12	1.56	1.56	**13f**	50	50	50	50
9	0.78	0.39	0.39	0.39	**13g**	>50	25	12.5	6.25
11a	>50	12.5	12.5	12.5					
11b	>50	25	25	25	Metronidazole	50	12.5	12.5	12.5

previous observations on structure–activity relationships [11]. In fact this activity resulted significant when a phenylthio (**5b,d,e**) group or a chlorine atom (**9**) in C-2 of the quinoxaline 1,4-dioxide system was present. Furthermore, a *para* fluorine atom (**5b**), a *para* methoxy group (**5d**) or both *meta* and *para* methoxy group (**5e**) on the phenylthio moiety were more favorable than chlorine atoms or methoxy groups in different positions. Oxidation of sulfur bridge to yield phenylsulfinyl and phenylsulfonyl derivatives or its replacement with benzylamino or phenylamino group slightly reduces this activity.

About anti-trichomonas assay, the results obtained are reported in Table 3. The quinoxaline 1,4-dioxides tested exhibited a general good activity against *T. vaginalis*. Compounds **5a,c,d, 7a, 8a–e, 9, 11c**, and **13b** showed MCs ⩽ 3.12 μg/ml. In particular compounds **5a,d, 7a, 9** and **11c** exhibited the best activity (MIC = 0.39–0.78 μg/ml). Also in this case the data seem to confirm that the sulfur atom in C-2 position is favorable for anti-trichomonas activity. Mono-oxidation of phenylthio moiety to phenylsulfinyl derivative or substitution with 3,4-dichlorobenzylamino group retains the biological activity, whereas di-oxidation to phenylsulfonyl derivative or substitution with phenylamino group reduces this activity. About the substituents on the phenylthio moiety, no substituent or a *para* methoxy group resulted the most favorable.

5. Conclusions

In the light of the results obtained from the screening of the phenyl substituted 6,7-difluoro-3-methyl-2-phenylthio/phenylsulfinyl/phenylsulfonyl/benzylamino/phenylamino-quinoxaline 1,4-dioxides synthesized, we can confirm that this scaffold is certainly endowed with anti-tubercular and anti-candida activities, associating for some quinoxaline 1,4-dioxides a moderate anti-mycoplasma activity against *M. hominis*. In addition, a very interesting activity emerged against *T. vaginalis* for compounds **5a,d, 7a, 9** and **11c** (MIC = 0.39–0.78 μg/ml) which were much more potent than

reference drug (Metronidazole). This observation may promote a further development of our research in this field.

6. Experimental

6.1. Chemistry

Melting points (m.p.) were uncorrected and were taken in open capillaries in a Digital Electrothermal IA9100 melting point apparatus. ^{1}H NMR spectra were recorded on a Varian XL-200 (200 MHz) instrument, using TMS as internal standard. The chemical shift values are reported in ppm (δ) and coupling constants (J) in Hertz (Hz). Signal multiplicities are represented by: s (singlet), d (doublet), dd (double doublet), m (multiplet), and br s (broad singlet). MS spectra of acetonylphenyl sulfides (**3a–e**) were performed on a combined HP 5790 (GC)–HP 5970 (MS) apparatus, while the products (**5b–e, 7a, 8b–e, 11a–g** and **13a–g**) were performed with a combined Liquid Chromatograph-Agilent 1100 series Mass Selective Detector (MSD). Column chromatography was performed using 70–230 mesh (Merck silica gel 60). Light petroleum refers to the fraction with b.p. 40–60 °C. The progress of the reactions, the R_f and the purity of the final compounds were monitored by TLC using Merck F-254 commercial plates. Analyses indicated by the symbols of the elements were within ±0.4% of the theoretical values.

6.1.1. Starting materials and intermediates

Thiophenol derivatives (**1a–e**), chloroacetone (**2**), benzylamine derivatives (**10a–g**) and aniline derivatives (**12a–g**) were commercially available. Acetonylphenyl sulfide (**3a**), 5,6-difluorobenzofuroxan (**4**), 6,7-difluoro-3-methyl-2-phenylthioquinoxaline 1,4-dioxide (**5a**), 6,7-difluoro-3-methyl-2-fenylsulfonylquinoxaline 1,4-dioxide (**8a**) and 2-chloro-6,7-difluoro-3-methylquinoxaline 1,4-dioxide (**9**) were known and prepared as previously described [11].

6.1.2. General procedure for preparation of acetonylphenyl sulfides (*3a–e*)

A solution of the appropriate thiophenol derivatives (**1a–e**) (6.0 mmol) and chloroacetone (**2**) (7.8 mmol) in pyridine (5 ml) was heated to 100 °C for 1 h. On cooling to room temperature (r.t.), the reaction mixture was added to 6 N hydrochloric acid solution (20 ml), then extracted with dichloromethane and dried on anhydrous sodium sulfate. On evaporation of the liquor mother, a yellow oil corresponding to acetonylphenyl sulfides (**3a–e**) was collected and the analytical properties are reported below.

*6.1.2.1. Acetonyl-4-fluorophenyl sulfide (**3b**).* This compound was obtained in 89% yield; ^{1}H NMR ($CDCl_3$): δ 7.40–7.33 (2H, m, H-3 + H-5), 7.05–6.96 (2H, m, H-2 + H-6), 3.61 (2H, s, CH_2), 2.27 (3H, s, CH_3). MS: *m/z* 184 $[M]^+$. Anal. C_9H_9FOS (C, H, N).

*6.1.2.2. Acetonyl-3,4-dichlorophenyl sulfide (**3c**).* This compound was obtained in 95% yield; ^{1}H NMR ($CDCl_3$): δ 7.42 (1H, d, J = 2.2, H-2), 7.36 (1H, d, J = 8.2, H-5), 7.15 (1H, dd, J = 8.2 and 2.2, H-6), 3.69 (2H, s, CH_2), 2.29 (3H, s, CH_3). MS: *m/z* 238, 236, 234 $[M]^+$. Anal. $C_9H_8Cl_2OS$ (C, H, N).

*6.1.2.3. Acetonyl-4-methoxyphenyl sulfide (**3d**).* This compound was obtained in 95% yield; ^{1}H NMR ($CDCl_3$): δ 7.35 (2H, d, J = 8.6, H-2 + H-6), 6.84 (2H, d, J = 8.6, H-3 + H-5), 3.79 (3H, s, OCH_3), 3.55 (2H, s, CH_2), 2.26 (3H, s, CH_3). MS: *m/z* 196 $[M]^+$. Anal. $C_{10}H_{12}O_2S$ (C, H, N).

*6.1.2.4. Acetonyl-3,4-dimethoxyphenyl sulfide (**3e**).* This compound was obtained in 73% yield; ^{1}H NMR ($CDCl_3$): δ 7.00–6.94 (2H, m, H-2 + H-6), 6.80 (1H, d, J = 8.2, H-5), 3.87 (6H, s, $2OCH_3$), 3.59 (2H, s, CH_2), 2.27 (3H, s, CH_3). MS: *m/z* 226 $[M]^+$. Anal. $C_{11}H_{14}O_3S$ (C, H, N).

6.1.3. General procedure for preparation of 6,7-difluoro-3-methyl-2-phenylthioquinoxaline 1,4-dioxides (*5a–e*)

The title compounds were prepared modifying the known Beirut reaction. Equimolar amounts (3.0 mmol) of the appropriate acetonylphenyl sulfides (**3a–e**) and 5,6-difluorobenzofuroxan (**4**) [11] were added to methanol (20 ml) and methanol saturated with dry ammonia gas (5 ml), then stirred at r.t. for 2 h. The resulting precipitate was filtered off, washed with little portions of cold methanol and dried, to afford the desired derivatives **5a–e**.

*6.1.3.1. 6,7-Difluoro-3-methyl-2-(4-fluorophenylthio)quinoxaline 1,4-dioxide (**5b**).* This compound was obtained in 39% yield; m.p. 161–162 °C (ethanol); TLC (light petroleum–ethyl acetate 70:30): R_f 0.15; ^{1}H NMR ($CDCl_3$): δ 8.47–8.28 (2H, m, H-5 + H-8), 7.50–7.43 (2H, m, H-3′ + H-5′), 7.09–7.00 (2H, m, H-2′ + H-6′), 2.91 (3H, s, CH_3). LC/MS: 339 [M + H], 361 [M + Na]. Anal. $C_{15}H_9F_3N_2O_2S$ (C, H, N).

*6.1.3.2. 6,7-Difluoro-3-methyl-2-(3,4-dichlorophenylthio)-quinoxaline 1,4-dioxide (**5c**).* This compound was obtained in 31% yield; m.p. 191–192 °C (ethanol); TLC (light petroleum–ethyl acetate 70:30): R_f 0.15; ^{1}H NMR ($CDCl_3$): δ 8.50–8.27 (2H, m, H-5 + H-8), 7.45 (1H, s, H-2′) 7.41 (1H, d, J = 8.8, H-5′), 7.24 (1H, d, J = 8.8, H-6′), 2.93 (3H, s, CH_3). LC/MS: 411 [M + Na]. Anal. $C_{15}H_8Cl_2F_2N_2O_2S$ (C, H, N).

*6.1.3.3. 6,7-Difluoro-3-methyl-2-(4-methoxyphenylthio)quinoxaline 1,4-dioxide (**5d**).* This compound was obtained in 39% yield; m.p. 166–167 °C (ethanol); TLC (light petroleum–ethyl acetate 1:1): R_f 0.48; ^{1}H NMR ($CDCl_3$): δ 8.46–8.30 (2H, m, H-5 + H-8), 7.45 (2H, d, J = 8.8, H-2′ + H-6′), 6.87 (2H, d, J = 8.8, H-3′ + H-5′), 3.80 (3H, s, OCH_3), 2.86 (3H, s, CH_3). LC/MS: 351 [M + H], 373 [M + Na], 389 [M + K]. Anal. $C_{16}H_{12}F_2N_2O_3S$ (C, H, N).

*6.1.3.4. 6,7-Difluoro-3-methyl-2-(3,4-dimethoxyphenylthio-)quinoxaline 1,4-dioxide (**5e**).* This compound was obtained in 46% yield; m.p. 182–184 °C (ethanol); TLC (light petro-

leum–ethyl acetate 70:30): R_f 0.14; ^{1}H NMR ($CDCl_3$): δ 8.47–8.31 (2H, m, H-5 + H-8), 7.06 (1H, s, H-2′), 7.04 (1H, d, J = 8.0, H-6′), 6.81 (1H, d, J = 8.0, H-5′), 3.87 (6H, s, 2OCH_3), 2.86 (3H, s, CH_3). LC/MS: 381 [M + H], 403 [M + Na]. Anal. $C_{17}H_{14}F_2N_2O_4S$ (C, H, N).

*6.1.4. General procedure for preparation of 6,7-difluoro-3-methyl-2-phenylsulfinylquinoxaline 1,4-dioxide (**7a**) and 6,7-difluoro-3-methyl-2-phenylsulfonylquinoxaline 1,4-dioxides (**8a–e**)*

The title compounds were prepared following the procedure previously described by Abushanab [15]. To a solution of the appropriate 6,7-difluoro-3-methyl-2-phenylthioquinoxaline 1,4-dioxide (**5a–e**) (3.0 mmol) in chloroform (40 ml) a solution of 3-chloroperoxybenzoic acid (MCPBA) in 1:2.5 molar ratio (7.5 mmol) in chloroform (15 ml) was slowly added. After the addition was complete, the reaction mixture was stirred at r.t. for an additional time as indicated below, then the chloroform solution was washed with 5% sodium bicarbonate aqueous solution, dried over anhydrous sodium sulfate, filtered off and evaporated. A solid corresponding to derivatives **8a–e** was obtained and purified as indicated below. Compound **7a** was in turn obtained in similar conditions but using 6,7-difluoro-3-methyl-2-phenylthioquinoxaline 1,4-dioxide (**5a**) (3.0 mmol) and MCPBA (3.6 mmol) in 1:1.2 molar ratio.

*6.1.4.1. 6,7-Difluoro-3-methyl-2-phenylsulfinylquinoxaline 1,4-dioxide (**7a**).* This compound was obtained in 50% yield after stirring for 5 h and crystallization from ethanol; m.p. 187–188 °C; TLC (light petroleum–ethyl acetate 80:20): R_f 0.17; ^{1}H NMR ($CDCl_3$): δ 8.44–8.25 (2H, m, H-5 + H-8), 8.09–8.04 (2H, m, H-2′ + H-6′), 7.56–7.53 (3H, m, H-3′ + H-4′ + H-5′), 2.95 (3H, s, CH_3). LC/MS: 359 [M + Na], 375 [M + K]. Anal. $C_{15}H_{10}F_2N_2O_3S$ (C, H, N).

*6.1.4.2. 6,7-Difluoro-3-methyl-2-(4-florophenylsulfonyl)quinoxaline 1,4-dioxide (**8b**).* This compound was obtained in 91% yield after stirring for 10 h; m.p. 117–119 °C (ethanol); TLC (light petroleum–ethyl acetate 70:30): R_f 0.15; ^{1}H NMR ($CDCl_3$): δ 8.43 (1H, m, H-8), 7.89 (1H, m, H-5), 7.68–7.54 (4H, m, 4 phenyl H), 3.21 (3H, s, CH_3). LC/MS: 371 [M + H]. Anal. $C_{15}H_9F_3N_2O_4S$ (C, H, N).

*6.1.4.3. 6,7-Difluoro3-methyl-2-(3,4-dichlorophenylsulfonyl)quinoxaline 1,4-dioxide (**8c**).* This compound was obtained in 80% yield after stirring for 15 h; m.p. 123–126 °C (ethanol); TLC (light petroleum–ethyl acetate 70:30): R_f 0.15; ^{1}H NMR ($CDCl_3$): δ 8.42 (1H, m, H-8), 8.20 (1H, m, H-5), 8.18 (1H, d, J = 2.2, H-2′), 7.68–7.46 (2H, m, H-3′ + H-5′), 3.18 (3H, s, CH_3). LC/MS: 421 [M + H], 459 [M + K]. Anal. $C_{15}H_8Cl_2F_2N_2O_4S$ (C, H, N).

*6.1.4.4. 6,7-Difluoro-3-methyl-2-(4-methoxyphenylsulfonyl)quinoxaline 1,4-dioxide (**8d**).* This compound was obtained in 82% yield after stirring for 5 h; m.p. 142–144 °C (ethanol); TLC (light petroleum–ethyl acetate 1:1): R_f 0.48; ^{1}H NMR ($CDCl_3$): δ 8.40 (1H, m, H-8), 8.23 (1H, m, H-5), 8.11 (2H, d, J = 8.8, H-2′ + H-6′), 7.03 (2H, d, J = 8.8, H-3′ + H-5′), 3.89 (3H, s, OCH_3), 3.19 (3H, s, CH_3). LC/MS: 405 [M + Na]. Anal. $C_{16}H_{12}F_2N_2O_5S$ (C, H, N).

*6.1.4.5. 6,7-Difluoro-3-methyl-2-(3,4-dimethoxyphenylsulfonyl)quinoxaline 1,4-dioxide (**8e**).* This compound was obtained in 85% yield after stirring for 5 h; m.p. 169–170 °C (ethanol); TLC (light petroleum–ethyl acetate 70:30): R_f 0.14; ^{1}H NMR ($CDCl_3$): δ 8.43 (1H, m, H-8), 8.24 (1H, m, H-5), 7.75 (1H, dd, J = 8.4 and 2.2, H-6′), 7.66 (1H, d, J = 2.2, H-2′), 6.98 (1H, d, J = 8.4, H-5′), 4.00 (3H, s, OCH_3), 3.96 (3H, s, OCH_3), 3.20 (3H, s, CH_3). LC/MS: 413 [M + H], 435 [M + Na], 451 [M + K]. Anal. $C_{17}H_{14}F_2N_2O_6S$ (C, H, N).

*6.1.5. General procedure for preparation of 6,7-difluoro-3-methyl-2-benzylaminoquinoxaline 1,4-dioxides (**11a–g**) and 6,7-difluoro-3-methyl-2-phenylaminoquinoxaline 1,4-dioxides (**13a–g**)*

Equimolar amounts (1.0 mmol) of 2-chloro-6,7-difluoro-3-methylquinoxaline 1,4-dioxide (**9**) [11], and the appropriate benzylamine (**10a–g**) or phenylamine (**12a–g**) in dry DMF (6 ml) were heated to 70 °C under stirring for 14 h. On cooling to r.t., the reaction mixture was diluted with 50 ml of water. A crude precipitate was formed and collected by filtration.

Purification of all compounds was carried out by crystallization from aqueous ethanol.

*6.1.5.1. 6,7-Difluoro-3-methyl-2-benzylaminoquinoxaline 1,4-dioxide (**11a**).* This compound was obtained in 62% yield; m.p. 102–104 °C (aqueous ethanol); TLC (light petroleum–ethyl acetate 1:1): R_f 0.22; ^{1}H NMR ($CDCl_3$): δ 8.18 (1H, m, H-8), 7.62 (1H, m, H-5), 7.50–7.30 (5H, m, 5 phenyl H), 5.18 (1H, br s, NH), 4.53 (2H, d, J = 4.8, CH_2), 2.79 (3H, s, CH_3). LC/MS: 356 [M + K]. Anal. $C_{16}H_{13}F_2N_3O_2$ (C, H, N).

*6.1.5.2. 6,7-Difluoro-3-methyl-2-(4-fluorobenzylamino)quinoxaline 1,4-dioxide (**11b**).* This compound was obtained in 59% yield; m.p. 180–181 °C (aqueous ethanol); TLC (light petroleum–ethyl acetate 40:60): R_f 0.24; ^{1}H NMR ($CDCl_3$): δ 7.60 (1H, m, H-8), 7.41–734 (3H, m, H-5 + H-3′ + H-5′), 7.13–7.04 (2H, m, H-2′ + H-6′), 5.16 (1H, br s, NH), 4.51 (2H, d, J = 5.4, CH_2), 2.78 (3H, s, CH_3). LC/MS: 336 [M + H], 358 [M + Na]. Anal. $C_{16}H_{12}F_3N_3O_2$ (C, H, N).

*6.1.5.3. 6,7-Difluoro-3-methyl-2-(3,4-dichlorobenzylamino) quinoxaline 1,4-dioxide (**11c**).* This compound was obtained in 73% yield; m.p. 194–196 °C (aqueous ethanol); TLC (light petroleum–ethyl acetate 30:70): R_f 0.27; ^{1}H NMR ($CDCl_3$): δ 8.20 (1H, m, H-8), 7.56–7.22 (4H, m, H-5 + 3 phenyl H), 5.26 (1H, br s, NH), 4.53 (2H, d, J = 4.8, CH_2),

2.78 (3H, s, CH_3). LC/MS: 386 [M + H]. Anal. $C_{16}H_{11}Cl_2F_2N_3O_2$ (C, H, N).

*6.1.5.4. 6,7-Difluoro-3-methyl-2-(4-methoxybenzylamino) quinoxaline 1,4-dioxide (**11d**).* This compound was obtained in 78% yield; m.p. 83–85 °C (aqueous ethanol); TLC (light petroleum–ethyl acetate 1:1): R_f 0.16; ^{1}H NMR ($CDCl_3$): δ 8.18 (1H, m, H-8), 7.62 (1H, m, H-5), 7.32 (2H, d, J = 7.8, H-2′ + H-6′), 6.91 (2H, d, J = 7.8, H-3′ + H-5′), 5.12 (1H, br s, NH), 4.45 (2H, d, J = 4.8, CH_2), 3.81 (3H, s, OCH_3), 2.78 (3H, s, CH_3). LC/MS: 386 [M + K]. Anal. $C_{17}H_{15}F_2N_3O_3$ (C, H, N).

*6.1.5.5. 6,7-Difluoro-3-methyl-2-(3,4-dimethoxybenzylamino)quinoxaline 1,4-dioxide (**11e**).* This compound was obtained in 53% yield; m.p. 103–105 °C (aqueous ethanol); TLC (light petroleum–ethyl acetate 70:30): R_f 0.18; ^{1}H NMR ($CDCl_3$): δ 8.18 (1H, m, H-8), 7.63 (1H, m, H-5), 6.98–6.84 (3H, m, 3 phenyl H), 5.17 (1H, br s, NH), 4.45 (2H, d, J = 4.8, CH_2), 3.89 (6H, s, 2OCH_3), 2.78 (3H, s, CH_3). LC/MS: 416 [M + K]. Anal. $C_{18}H_{17}F_2N_3O_4$ (C, H, N).

*6.1.5.6. 6,7-Difluoro-3-methyl-2-(3,5-dimethoxybenzylamino)quinoxaline 1,4-dioxide (**11f**).* This compound was obtained in 62% yield; m.p. 196–197 °C (aqueous ethanol); TLC (light petroleum–ethyl acetate 70:30): R_f 0.25; ^{1}H NMR ($CDCl_3$): δ 8.18 (1H, m, H-8), 7.60 (1H, m, H-5), 6.51 (2H, s, H-2′ + H-6′), 6.40 (1H, s, H-4′), 5.17 (1H, br s, NH), 4.46 (2H, d, J = 4.8, CH_2), 3.79 (6H, s, 2OCH_3), 2.78 (3H, s, CH_3). LC/MS: 416 [M + K]. Anal. $C_{18}H_{17}F_2N_3O_4$ (C, H, N).

*6.1.5.7. 6,7-Difluoro-3-methyl-2-(3,4,5-trimethoxybenzylamino)quinoxaline 1,4-dioxide (**11g**).* This compound was obtained in 61% yield; m.p. 193–194 °C (aqueous ethanol); TLC (chloroform–methanol 95:5): R_f 0.45; ^{1}H NMR ($CDCl_3$): δ 8.20 (1H, m, H-8), 7.64 (1H, m, H-5), 6.62 (2H, s, H-2′ + H-6′), 5.15 (1H, br s, NH), 4.45 (2H, d, J = 5.0, CH_2), 3.88 (6H, s, C-3′ OCH_3 + C-5′ OCH_3), 3.85 (3H, s, C-4′ OCH_3), 2.79 (3H, s, CH_3). LC/MS: 408 [M + H], 430 [M + Na]. Anal. $C_{19}H_{19}F_2N_3O_5$ (C, H, N).

*6.1.5.8. 6,7-Difluoro-3-methyl-2-phenylaminoquinoxaline 1,4-dioxide (**13a**).* This compound was obtained in 81% yield; m.p. 230–231 °C (aqueous ethanol); TLC (light petroleum–ethyl acetate 1:1): R_f 0.40; ^{1}H NMR ($CDCl_3$): δ 8.67 (1H, br s, NH), 8.45–8.32 (2H, m, H-5 + H-8), 7.41–7.30 (3H, m, H-3′ + H-4′ + H-5′), 7.08–7.05 (2H, m, H-2′ + H-6′), 2.28 (3H, s, CH_3). LC/MS: 304 [M + H], 326 [M + Na]. Anal. $C_{15}H_{11}F_2N_3O_2$ (C, H, N).

*6.1.5.9. 6,7-Difluoro-3-methyl-2-(4-fluorophenylamino)quinoxaline 1,4-dioxide (**13b**).* This compound was obtained in 81% yield; m.p. 218–219 °C (aqueous ethanol); TLC (light petroleum–ethyl acetate 1:1): R_f 0.30; ^{1}H NMR ($CDCl_3$): δ 8.63 (1H, br s, NH), 8.45–8.32 (2H, m, H-5 + H-8), 7.11–7.07 (4H, m, 4 phenyl H), 2.27 (3H, s, CH_3). LC/MS: 322 [M + H], 344 [M + Na]. Anal. $C_{15}H_{10}F_3N_3O_2$ (C, H, N).

*6.1.5.10. 6,7-Difluoro-3-methyl-2-(3,4-dichlorophenylamino)quinoxaline 1,4-dioxide (**13c**).* This compound was obtained in 33% yield; m.p. 224–226 °C (aqueous ethanol); TLC (light petroleum–ethyl acetate 1:1): R_f 0.46; ^{1}H NMR ($CDCl_3$): δ 8.55 (1H, br s, NH), 8.47–8.32 (2H, m, H-5 + H-8), 7.45 (1H, d, J = 8.6, H-5′), 7.14 (1H, d, J = 2.4, H-2′), 6.89 (1H, dd, J = 8.6 and 2.4, H-6′), 2.35 (3H, s, CH_3). LC/MS: 410 [M + K]. Anal. $C_{15}H_9Cl_2F_2N_3O_2$ (C, H, N).

*6.1.5.11. 6,7-Difluoro-3-methyl-2-(4-methoxyphenylamino-)quinoxaline 1,4-dioxide (**13d**).* This compound was obtained in 74% yield; m.p. 203–204 °C (aqueous ethanol); TLC (light petroleum–ethyl acetate 1:1): R_f 0.44; ^{1}H NMR ($CDCl_3$): δ 8.71 (1H, br s, NH), 8.44–8.30 (2H, m, H-5 + H-8), 7.06 (2H, d, J = 8.8, H-2′ + H-6′), 6.91 (2H, d, J = 8.8, H-3′ + H-5′), 3.84 (3H, s, OCH_3), 2.25 (3H, s, CH_3). LC/MS: 334 [M + H], 356 [M + Na], 372 [M + K]. Anal. $C_{16}H_{13}F_2N_3O_3$ (C, H, N).

*6.1.5.12. 6,7-Difluoro-3-methyl-2-(3,4-dimethoxyphenylamino)quinoxaline 1,4-dioxide (**13e**).* This compound was obtained in 64% yield; m.p. 214–216 °C (aqueous ethanol); TLC (light petroleum–ethyl acetate 70:30): R_f 0.33; ^{1}H NMR ($CDCl_3$): δ 8.70 (1H, br s, NH), 8.44–8.30 (2H, m, H-5 + H-8), 6.86–6.67 (3H, m, 3 phenyl H), 3.91 (3H, s, OCH_3), 3.87 (3H, s, OCH_3), 2.28 (3H, s, CH_3). LC/MS: 364 [M + H], 386 [M + Na]. Anal. $C_{17}H_{15}F_2N_3O_4$ (C, H, N).

*6.1.5.13. 6,7-Difluoro-3-methyl-2-(3,5-dimethoxyphenylamino)quinoxaline 1,4-dioxide (**13f**).* This compound was obtained in 59% yield; m.p. 217–218 °C (aqueous ethanol); TLC (light petroleum–ethyl acetate 1:1): R_f 0.39; ^{1}H NMR ($CDCl_3$): δ 8.55 (1H, br s, NH), 8.45–8.33 (2H, m, H-5 + H-8), 6.34 (1H, m, H-4′), 6.19 (2H, s, H-2′ + H-6′), 3.78 (6H, s, 2OCH_3), 2.37 (3H, s, CH_3). LC/MS: 364 [M + H], 386 [M + Na]. Anal. $C_{17}H_{15}F_2N_3O_4$ (C, H, N).

*6.1.5.14. 6,7-Difluoro-3-methyl-2-(3,4,5-trimethoxyphenylamino)quinoxaline 1,4-dioxide (**13g**).* This compound was obtained in 85% yield; m.p. 198–199 °C (aqueous ethanol); TLC (light petroleum–ethyl acetate 40:60): R_f 0.22; ^{1}H NMR ($CDCl_3$): δ 8.62 (1H, br s, NH), 8.45–8.32 (2H, m, H-5 + H-8), 6.31 (2H, s, H-2′ + H-6′), 3.87 (3H, s, $C_{4'}$–OCH_3), 3.82 (6H, s, $C_{3'}$–OCH_3 + $C_{5'}$–OCH_3), 2.35 (3H, s, CH_3). LC/MS: 3.94 [M + H], 416 [M + Na]. Anal. $C_{18}H_{17}F_2N_3O_5$ (C, H, N).

6.2. Microbiological assays

6.2.1. Antibacterial assays

The strains used in these tests were from American Type Culture Collection (ATCC): *S. aureus* ATCC 2913, *E. coli* ATCC 25922, *K. pneumoniae* ATCC 700603, and *P. aeruginosa* ATCC 27853, or were environmental isolate (*V. alginolyticus*). A logarithmic phase culture of each bacterial strain was diluted with Luria broth in order to obtain a density of 10^6 CFU/ml. The test was performed in a 96-well

microtiter plate in a final volume of 100 µl. Test compounds were dissolved in dimethyl sulfoxide at an initial concentration of 1000 µg/ml and serially diluted in the plate (500–7.8 µg/ml) using Luria broth. Each well was then inoculated with the standardized bacterial suspension and incubated at 37 °C for 18–24 h. One well containing bacteria without sample (growth control, GC), and one well containing broth only (sterility control) were also used. After the incubation the growth (or its lack) of the bacteria was determined visually in both containing compound well and control well. The lowest concentration at which there was no visible growth (turbidity) was taken as the MIC. In addition 5 µl of suspension from each well were inoculated in a Mueller Hinton agar plate to control bacterial viability.

6.2.2. Anti-candida assay

Antifungal activity was determined by the tube dilution method on four clinical isolates of *Candida* species (*C. albicans, C. glabrata, C. krusei* and *C. parapsilosis*) coming from patients with AIDS and candidemia. Yeast inocula were obtained by properly diluting cultures incubated at 35 °C for 48 h in Sabouraud Dextran agar to obtain a density of 10^6 CFU/ml. Test compounds were dissolved in dimethyl sulfoxide at an initial concentration of 1000 µg/ml and then were serially diluted in Brain Hearth fusion broth to 0.1 µg/ml. Then, 0.5 ml of the above serial dilutions of test compounds were added, in sterile polystyrene tubes, with an equal volume of fungal suspension and incubated at 35 °C for 48 h. Preliminary tests with dimethyl sulfoxide were performed to assure that no *Candida* species inhibition occurred at used concentrations.

The MIC_{100} determination was performed in duplicate, and defined as the lowest concentration of the compound which produced no visible growth. MFC determinations were done for all compounds by plating 20 µl of each well on Sabouraud dextrose agar (SDA) plates after 48 h of incubation at 35 °C. The MFC was the lowest drug concentration giving no growth on the (SDA) plates (>98% killing). A sample of compound-free GC and a set of tubes with sample alone for monitoring contamination of the medium were used.

6.2.3. Anti-mycobacterial assay

Anti-mycobacterial activity was determined by the new method Bactec MGIT 960 [16,17] against *M. tuberculosis* H37Rv (ATCC 27294). A suspension of micro-organisms was prepared in 7H9 medium at a density of 0.5 Mc Farland and was diluted (1:5) with sterile saline solution. Test compounds were dissolved in dimethyl sulfoxide at an initial concentration of 2240 mg/ml and diluted to obtain a final concentration of 8, 4, 2 and 1 µg/ml (rifampin was used as reference drug). To each 7 ml MGIT tube, 0.8 ml of MGIT 960 growth supplement and 0.1 ml of the test compound were aseptically added, and finally 0.5 ml of the test inoculum was added.

For each isolate a GC tube with growth supplement and without test compound was included. For this GC, the inoculum was prepared by pipetting 0.1 ml of the test inoculum with 10 ml of sterile saline to make a 1:100 dilution and 0.5 ml added to the MIGT tube. All the inoculated tubes were placed into the Bactec MGIT 960 instrument the same day of inoculation.

The relative growth ratio between the compound-containing tube and the compound-free GC tube was determined by the system's software algorithm. If the relative growth in the compound-containing tube was equal to or exceeded that of the GC tube, the isolate was considered compound resistant; if the relative growth was less than that of the GC tube, the isolate was considered compound susceptible.

6.2.4. Anti-mycoplasma assay

M. hominis used for this assay was a clinical isolate strain and was grown on BEa broth [18]. The test was performed in a 96-well microtiter plate in a concentration of bacteria in BEa broth of 10^6 bacteria per well. Test compounds were dissolved in dimethyl sulfoxide at an initial concentration of 10 mg/ml and serially diluted to 0.1 µg/ml, then added at the plate. Each well was then incubated at 37 °C in humid atmosphere containing 5% CO_2. The MBC determination was defined as the lowest concentration of the compound, which did not produce color change of the broth, after removal of test compound, the bacteria showed no growth. Wells containing bacteria on BEa broth and the same amount of dimethyl sulfoxide, without sample (GC), were also used. In these cases each well produced color change indicating the bacterial growth. Gentamicine used as control drug showed MBC = 5–25 µg/ml in different experiments.

6.2.5. Anti-trichomonas assay

Our study was performed by using *T. vaginalis* (SS-22), *Mycoplasma*-free, isolated in Sassari (Italy) from a case of acute vaginal trichomoniasis, axenically grown at 37 °C in Diamond medium [19] supplemented with 10% heat-inactivated fetal calf serum (FCS). *Trichomonas* were used immediately after isolation, or frozen in liquid nitrogen. Stock solutions (2 mg/ml) of metronidazole (SIGMA) and other compounds tested were prepared in dimethyl sulfoxide, and stored in small aliquots at 4 °C.

The susceptibility of *T. vaginalis* was determined by the assessment of growth and motility of flagellates exposed to different concentrations of drugs. Tests were performed in sterile tissue-culture microtiter (Corning). Serial twofold dilution of the compounds were made in Diamond medium containing 10% FCS. Control wells received 100 µl of complete Diamond medium. Protozoa, washed twice in phosphate-buffered saline (PBS, pH 7.2), were suspended in the same medium at the density of 2.5×10^5 cells per ml and 100 µl of the calibrate suspension was added to each well. Final concentrations obtained ranged from 50 to 0.006 µg/ml. Tests were performed in duplicate and repeated at least three times. Microtiter plates were then incubated aerobically at 37 °C, in a humid atmosphere containing 5%

CO_2. At 24, 48, 72 and 96 h of incubation, plates were examined with an inverted microscope (OLIMPUS CK) and checked for protozoan motility. The lowest concentration in which no motile flagellates were seen was defined as MIC_{100}, according to Meingassner and Thurner [20]. Preliminary tests with dimethyl sulfoxide were performed to assure that no *T. vaginalis* inhibition occurred at used concentrations.

In all experimental conditions control cells were viable and motile. The susceptibility of the isolate SS-22 to metronidazole, used as reference drug, was scanty (MIC_{100} of 50, 12.5 μg/ml, respectively, at 24 h of incubation and next times).

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