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THE ANTIFUNGAL ACTIVITY OF SULFONYLATED/CARBOXYLATED DERIVATIVES OF DIBENZO-1,4-DIOXINE-2-ACETYLOXIME MAY BE DUE TO INHIBITION OF LANOSTEROL-14α-DEMETHYLASE

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Aryl/alkyl-sulfonyl-, aryl/alkylcarboxyl- and aryl(sulfonyl)carbamyl/thiocarbamyl-derivatives of dibenzo-1,4-dioxine-2-acetyloxime were prepared by reaction of the title compound with sulfonyl halides, sulfonic acid anhydrides, acyl chlorides/carboxylic acids, arylsulfonyl isocyanates, aryl/acyl isocyanates or isothiocyanates. Several of the newly synthesized compounds showed effective *in vitro* antifungal activity against *Aspergillus* and *Candida spp.*, some of them showing activities comparable to ketoconazole (with minimum inhibitory concentrations in the range of 1.2–4 μg/mL) against the two *Aspergillus* strains, but possessing a lower activity as compared to ketoconazole against *C. albicans*. Of the three investigated strains, best activity was detected against *A. flavus*. The mechanism of action of these compounds probably involves inhibition of ergosterol biosynthesis by interaction with lanosterol-14-α-demethylase (CYP51A1), since reduced amounts of ergosterol were found by means of HPLC, in cultures of the sensitive strain *A. flavus* treated with some of these inhibitors. Thus, the compounds reported here might possess a similar mechanism of action at molecular level with that of the widely used azole antifungals.

Keywords: Dibenzo-1,4-dioxine-2-acetyloxime; Sulfonylation; (Thio)carbamates; Antifungal compounds; Lanosterol-14-α-demethylase; Ergosterol biosynthesis inhibitors; Azole antifungals

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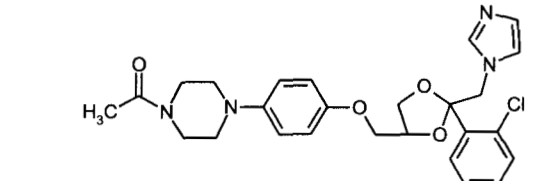
INTRODUCTION

Azole antifungals belonging to the first generation of such drugs, as for example ketoconazole **1** and itraconazole **2** among others, have revolutionized the treatment of serious systemic/topical fungal infections.[1–4] However, the treatment of many other fungal diseases, particularly aspergillosis, registered less success.[1] This fact, correlated with an increasing emergence of fungal resistance to the presently available drugs,[4–9] makes the requirement for new, broad-spectrum antifungal agents an important task for the drug designer.[10–15]



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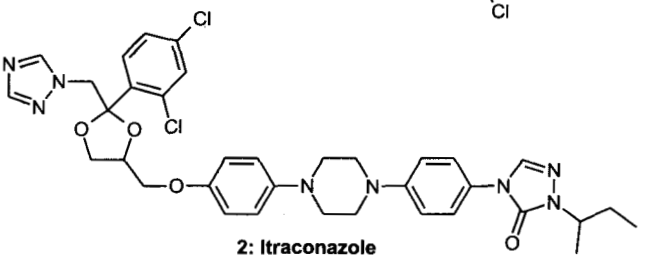

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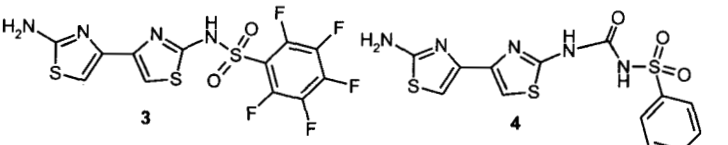
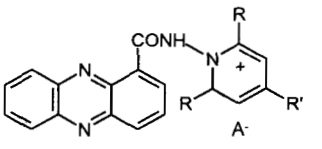


3 **4**

3, 4: MIC = 0.5 - 1 ug/mL
(*A. niger* and *A. flavus*)



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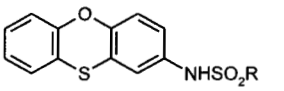

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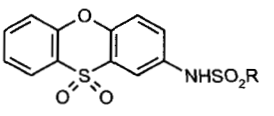

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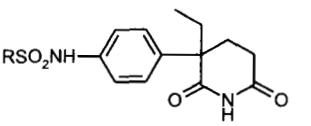

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In previous communications from this laboratory it was shown that alkyl/arylsulfonylated-, arylsulfonylureido- or pyridinium-based derivatives of polycyclic ring systems, of types **3–8**, possess interesting antifungal action against different *Aspergillus* and *Candida albicans* strains.[16,17] Furthermore, several metal complexes of these and other types of sulfonylated ligands showed improved properties.[18–20] All this data prompted us to investigate some other types of such derivatives for their possible antifungal activity. Here we report the synthesis of some new aryl/alkyl-sulfonyl-, aryl/alkyl-carboxyl- and carbamyl/thiocarbamyl derivatives of dibenzo-1,4-dioxine-2-acetyloxime which were obtained by reaction of the title compound with sulfonyl halides/sulfonic acid anhydrides, carboxylic acids/acyl chlorides, arylsulfonyl isocyanates, aryl/acyl isocyanates or isothiocyanates. Several of the newly synthesized compounds showed effective *in vitro* antifungal activity against two *Aspergillus* and one *Candida spp.*, some of them possessing activities comparable to ketoconazole (with minimum inhibitory concentrations (MIC-s) in the range of 1.2–4 μg/mL) against the two aspergilli, but being much less active than ketoconazole against *Candida*. The possible mechanism of antifungal action with these new derivatives has also been investigated.

MATERIALS AND METHODS

Melting points were obtained with a heating plate microscope and are uncorrected. Elemental analyses (C, H, N) were done by combustion with a Carlo Erba Instrument and were ± 0.4% of the theoretical values, calculated for the proposed formula. IR spectra were recorded with a Perkin-Elmer spectrophotometer in KBr pellets. ^{1}H-NMR spectra have been recorded at 200 MHz, with a Varian Gemini 200 spectrophotometer in solvents specified in each case; chemical shifts are reported as δ values relative to Me_4Si as standard. Reverse-phase HPLC was performed with a Beckman instrument, on a μ-Bondapak-C18 column, with acetonitrile as eluting solvent.

Dibenzo-1,4-dioxine-2-acetyloxime **9** was prepared by literature procedures.[21] Arylsulfonyl halides and isocyanates, aryl isocyanates/isothiocyanates, carboxylic acids and their acyl halides, carbodiimides (EDCI.HCl; diisopropylcarbodiimide), solvents and other reagents used in the synthesis were commercially available (from Sigma-Aldrich, Fluka or Acros) and were used without further purification.

General Procedure for Preparation of the Arylsulfonyl Derivatives 10–35

The previously reported methods for introducing alkyl/arylsulfonyl moieties into the molecules of biologically active compounds have been adapted for the preparation of these derivatives.[16,17a-c,22] An amount of 240 mg (1 mMole) of **9** suspended in 50 mL of anhydrous acetonitrile was treated with the stoichiometric amount (1 mMole) of sulfonyl halide (chloride or fluoride) or sulfonic acid anhydride and magnetically stirred at room temperature for 15 min. The stoichiometric amount (1 mMole) of pyridine or triethylamine was then added to the reaction mixture, and stirring was continued at room temperature for 6–10 h (TLC control). The solvent was evaporated *in vacuo*, and the oil obtained poured into 100 mL of cold water. The precipitated sulfonylated derivative was filtered, dried and recrystallized from ethanol or methanol. Yields are shown in Table I.

General Procedure for Preparation of Carbamyl- and Arylsulfonylcarbamyl Derivatives 36–47

The previously reported methods for introducing (arylsulfonyl)ureido/carbamyl moieties into the molecules of biologically active compounds have been adapted for the preparation of these derivatives.[16,17a-c,23] An amount of 240 mg (1 mMole) of **9** was suspended in 100 mL of highly anhydrous acetonitrile (kept on molecular sieves) and magnetically stirred at 4°C for 10 min. The stoichiometric amount of arylsulfonyl isocyanate or arylisocyanate/isothiocyanate (eventually dissolved in the same solvent for the solid compounds or in pure state for the liquid ones) was then added dropwise, maintaining the temperature under 10°C when arylsulfonyl isocyanates were used, and boiling at reflux for the other derivatives. The reaction mixtures were then worked up as described above. Yields are shown in Table I.

General Procedure for Preparation of the Carboxy Derivatives 48–62

The previously reported methods for introducing aryl/alkylcarboxy moieties into the molecules of biologically active compounds have been adapted for the preparation of these derivatives.[16,17a-c,22,23] When acyl chlorides were used, the method described for the preparation of derivatives **10–35** was applied. When carboxylic acids was used, the method used was as follows: an amount of 720 mg (3 mMoles) of **9** was dissolved in 50 mL of anhydrous acetonitrile and then treated with the stoichiometric amount (3 mMoles) of

carboxylic acid. An amount of 570 mg (3 mMoles) of EDCI · HCl was then added and the reaction mixture was magnetically stirred at room temperature for 15 min, then 90 μL (6 mMoles) of triethylamine was added and stirring was continued for 8–10 h at 4°C (TLC control). The solvent was evaporated *in vacuo* and the residue taken up in 100 mL of water, the precipitated derivative **48–62** filtered, dried and recrystallized from EtOH or MeOH. Yields are shown in Table I.

Spectral and analytical data for some representative compounds of each series of new derivatives are presented below.

Dibenzo-1,4-dioxine-2-acetyloxime 2-nitrobenzenesulfonate **23**, as yellow crystals, mp 185–6°C. IR (KBr), cm^{-1}: 1139 (SO_2^{sym}), 1350 (SO_2^{as}), 1620 (C=N); ^{1}H-NMR (DMSO-d_6), δ, ppm: 2.01 (s, 3H, Me), 6.95–7.80 (m, 11H, ArH). Found: C, 56.29; H, 3.51; N, 6.50. $C_{20}H_{14}N_2O_7S$ requires: C, 56.34; H, 3.31; N, 6.57%.

Dibenzo-1,4-dioxine-2-acetyloxime 2-carboxybenzenesulfonate **31**, as tan crystals, mp 212–3°C. IR (KBr), cm^{-1}: 1176 (SO_2^{sym}), 1339 (SO_2^{as}), 1620 (C=N), 1730 (COOH); ^{1}H-NMR (DMSO-d_6), δ, ppm: 2.00 (s, 3H, Me), 7.02–7.94 (m, 11H, ArH), 10.12 (br s, 1H, COOH). Found: C, 59.47; H, 3.76; N, 3.08. $C_{21}H_{15}NO_7S$ requires: C, 59.29; H, 3.55; N, 3.29%.

Dibenzo-1,4-dioxine-2-acetyloxime 4-methoxybenzenesulfonate **33**, as tan crystals, mp 153–5°C. IR (KBr), cm^{-1}: 1176 (SO_2^{sym}), 1352 (SO_2^{as}), 1625 (C=N); ^{1}H-NMR (DMSO-d_6), δ, ppm: 2.01 (s, 3H, Me), 3.75 (s, 3H, MeO), 6.90–7.76 (m, 11H, ArH). Found: C, 61.09; H, 4.36; N, 5.98. $C_{21}H_{17}NO_6S$ requires: C, 61.31; H, 4.16; N, 6.10%.

Dibenzo-1,4-dioxine-2-acetyloxime 4-chlorobenzenesulfonylcarbamate **38**, as tan crystals, mp 234–5°C. IR (KBr), cm^{-1}: 1176 (SO_2^{sym}), 1285 (amide III), 1352 (SO_2^{as}), 1540 (amide II), 1625 (C=N), 3065 (NH); ^{1}H-NMR (DMSO-d_6), δ, ppm: 2.00 (s, 3H, Me), 6.98–7.80 (m, 7H, ArH from the dibenzodioxine moiety), 7.40–7.73 (m, AA′BB′, J_{AB} = 7.0 Hz, 4H, ArH, *p*-Cl-phenylene), 9.40 (br s, 1H, N*H*COO-). Found: C, 54.91; H, 3.06; N, 5.90. $C_{21}H_{15}ClN_2O_6S$ requires: C, 54.97; H, 3.29; N, 6.10%.

Dibenzo-1,4-dioxine-2-acetyloxime 3,4-dichlorophenylcarbamate **45**, as white crystals, mp 280–1°C. IR (KBr), cm^{-1}: 1277 (amide III), 1545 (amide II), 1625 (C=N), 3060 (NH); ^{1}H-NMR (DMSO-d_6), δ, ppm: 2.05 (s, 3H, Me), 7.01–7.82 (m, 10H, ArH), 9.04 (s, 1H, N*H*COO-). Found: C, 58.75; H, 3.05; N, 6.50. $C_{21}H_{14}Cl_2N_2O_4$ requires: C, 58.76; H, 3.29; N, 6.53%.

Dibenzo-1,4-dioxine-2-acetyloxime pentafluorocarboxylate **54**, as white crystals, mp 127–8°C. IR (KBr), cm^{-1}: 1625 (C=N), 1770 (COO); ^{1}H-NMR (DMSO-d_6), δ, ppm: 2.03 (s, 3H, Me), 7.01–7.74 (m, 7H, ArH). Found: C, 57.69; H, 2.50; N, 3.13. $C_{21}H_{10}F_5NO_4$ requires: C, 57.94; H, 2.32; N, 3.22%.

Assay of Fungistatic Activity of Compounds 10–62

Fungistatic activity was determined by a modification of the growth method previously reported by us,[16–20] utilizing two *Aspergillus* and one *Candida spp.* Minimum inhibitory concentrations (MICs) were determined by the agar dilution method with Iso-Sensitest agar as described by Kinsman *et al.*[24] The fungi/moulds were cultivated in agar plates at 37°C for 5 days in the nutrient broth (NB, Diagnostic Pasteur), in the absence and in the presence of test compounds. Stock solutions of inhibitors were obtained in DMSO (100 mg/mL) and dilutions up to 0.01 μg/mL were done with distilled deionized water. The minimum concentration at which no growth was observed was taken as the MIC value (μg/mL), and represents the mean of at least two determinations.

Assay of Sterols Present in the Fungi Cultures

A reverse-phase HPLC method adapted from the literature[25] was used to determine the amount of sterols (ergosterol and lanosterol) present in the fungi cultures. The fungi were cultivated as mentioned above for 5 days, with or without inhibitors added in the nutrient broth. Culture media were suspended in a small volume of MOPS buffer (pH 7.4) and the cells centrifuged at 20000 × g for 30 min. Cells were weighed (wet paste) and broken by sonication. Sterols present in the homogenate were then extracted in chloroform, the solvent was evaporated to a small volume and the extracts applied on a μ-Bondapak-C18 column, with acetonitrile as eluting solvent. Authentic ergosterol and lanosterol (from Sigma) were used as standards. The flow rate was 3 mL/min. The retention times were: 8.87 min for ergosterol and 7.62 min for lanosterol. Blank assays were done for cultures which were not treated with inhibitors in order to assess the normal levels of sterols present. The amount of ergosterol present in the same amount of wet cells from the culture grown in the absence of inhibitor was taken as 100%.[16–20,25,26]

RESULTS AND DISCUSSION

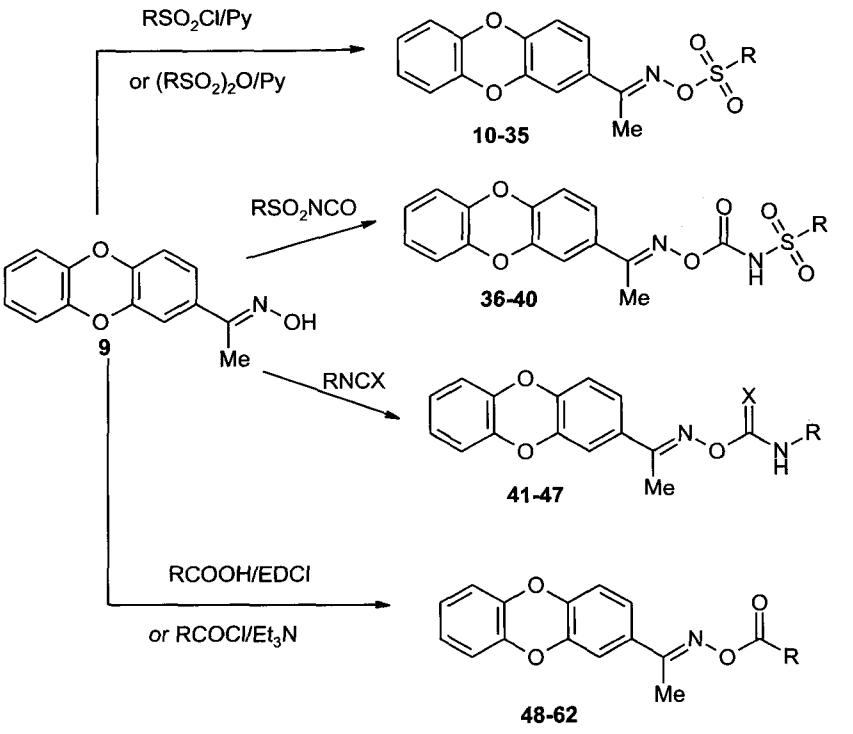
The new compounds, **10–62**, reported in the present study, were prepared by reaction of dibenzo-1,4-dioxine-2-acetyloxime, **9**, with arylsulfonyl halides/anhydrides, arylsulfonyl isocyanates, aryl isocyanates/isothiocyanates, carboxylic acids or acyl halides, and are shown in Table I.

The new derivatives, **10–62**, were obtained by routine procedures for the introduction of alkyl/arylsulfonyl, aryl(sulfonyl)ureido/carbamyl or alkyl/

TABLE I Derivatives **10–62** reported in the present study and their methods of preparation

Compound	*R*	*Yield (%)*	*Synthesis method*
10	CH_3-	85	A
11	CCl_3-	79	A
12	CF_3-	65	B
13	Me_2N-	26	A
14	$PhCH_2$-	44	C
15	Ph-	82	A
16	*p*-F-C_6H_4-	60	A
17	*p*-Cl-C_6H_4-	67	A
18	*p*-Br-C_6H_4-	69	A
19	*p*-I-C_6H_4-	75	A
20	*p*-CH_3-C_6H_4-	86	A
21	*p*-O_2N-C_6H_4-	90	A
22	*m*-O_2N-C_6H_4-	56	A
23	*o*-O_2N-C_6H_4-	60	A
24	3-Cl-4-O_2N-C_6H_3-	86	A
25	*p*-AcNH-C_6H_4-	67	A
26	*p*-H_2N-C_6H_4-	59	B
27	3-H_2N-4-MeO-C_6H_3-	45	A
28	C_6F_5-	88	A
29	*o*-HOOC-C_6H_4-	92	D
30	*m*-HOOC-C_6H_4-	85	A
31	*p*-HOOC-C_6H_4-	76	A
32	o-HOOC-C_6Br_4-	81	D
33	*p*-CH_3O-C_6H_4-	69	A
34	2,4,6-$(CH_3)_3$-C_6H_2-	83	A
35	*m*-CF_3-C_6H_4-	76	A
36	Ph-	95	E
37	*p*-F-C_6H_4-	94	E
38	*p*-Cl-C_6H_4-	89	E
39	*p*-CH_3-C_6H_4-	96	E
40	*o*-CH_3-C_6H_4-	95	E
41	*p*-F-C_6H_4- (X = O)	83	E
42	*p*-Cl-C_6H_4- (X = O)	85	E
43	*m*-Cl-C_6H_4- (X = O)	89	E
44	2,4-$F_2C_6H_3$ (X = O)	69	E
45	3,4-$Cl_2C_6H_3$ (X = O)	85	E
46	1-Naphthyl- (X = O)	73	E
47	PhCO- (X = S)	92	E
48	Ph_2N-	70	A
49	Pyridin-1-yl	51	A
50	Pyridin-2-yl	59	A
51	Pyridin-3-yl	67	A
52	CH_3-	84	A
53	CF_3-	25	A
54	C_6F_5-	40	A
55	*p*-H_2NO_2S-C_6H_4-	79	A
56	*p*-Pr_2NO_2S-C_6H_4-	85	A
57	*p*-Me_2N-C_6H_4-	64	A
58	*o*-F-C_6H_4-	70	A
59	*o*-Cl-C_6H_4-	65	A
60	*o*-I-C_6H_4-	90	A
61	*m*-I-C_6H_4-	76	A
62	*p*-I-C_6H_4-	85	A

A – **9** + RSO_2Cl (or RCOCl, or RCOOH/carbodiimide); B – **9** + triflic anhydride; C – **9** + RSO_2F; D – **9** + sulfobenzoic cyclic anhydride; E – **9** + RSO_2NCO (or RNCO, or RCONCS).



SCHEME 1 Synthesis of compounds **10–62**.

arylcarboxy moieties into the molecules of biologically active compounds, previously developed by our group,[16,17,20,22] as outlined in Scheme 1, and were characterized by spectroscopic and analytical data that confirmed their structures (elemental analysis data for C, H and N were ± 0.4% of the theoretical values, calculated for the proposed formulas; data are shown for some representative compounds in the Experimental section).

Antifungal activity data with the new derivatives, **10–62**, and the standard azole drug ketoconazole, **1**, are shown in Table II.

From the data of Table II, it should be noted that the new compounds, **10–62**, reported here represent a new class of antifungals with MIC-s (minimum inhibitory concentration) in the micromolar range, which might induce strong *in vivo* antifungal effects. The antifungal properties were strongly dependent on the substitution pattern at the modified acetoxime moiety, as follows: (i) aliphatic sulfonates (**10–13**) or carboxylates (**52, 53**) were ineffective antifungals, with MIC-s over 100 μg/mL, (ii) several of the prepared substituted-aryl sulfonates (such as **14–18, 20–22**) possess a modest efficacy as antifungals, with MIC-s in the range of 35–85 μg/mL, (iii) several

TABLE II Antifungal activity of compounds **10–62** against several organisms

Compound	*MIC** (μg/mL)		
	A. flavus C1150	*A. niger* C418	*Candida albicans* C316
Ketoconazole 1	1.2	1.8	0.06
10	>100	>100	85
11	>100	>100	76
12	90	>100	>100
13	>100	>100	>100
14	81	95	>100
15	83	85	86
16	95	83	84
17	61	92	87
18	35	76	80
19	13	14	13
20	55	59	75
21	40	46	51
22	36	37	43
23	13	15	27
24	18	18	23
25	19	21	25
26	5	7	6
27	3	6	8
28	2.0	2.5	3
29	4	6	5
30	6	7	7
31	8	9	10
32	3	3	5
33	19	21	25
34	14	16	16
35	6	7	8
36	1.5	1.5	1.9
37	1.4	1.8	2.0
38	1.2	1.7	2.2
39	1.3	1.9	2.7
40	1.5	1.8	3
41	1.9	3.1	3.5
42	2.4	3.2	5
43	2.1	2.5	7
44	1.5	2.3	3.4
45	1.4	1.6	2.9
46	1.6	1.7	2.3
47	1.2	1.9	2.7
48	10	13	13
49	6	7	10
50	6	10	16
51	4	9	8
52	>100	>100	>100
53	>100	>100	>100
54	6	7	13
55	3	4	7
56	2	2	3
57	4	7	8
58	14	15	16
59	13	17	18
60	9	11	15
61	6	8	6
62	4	5	8

* Errors are in the range of 5–10% (from 2 determinations).

other sulfonates, such as **19, 23–25, 33–35**, as well as some esters (**10**, **58–60**) showed effective antifungal properties, with MIC-s in the range of 9–25 μg/mL, (iv) effective antifungal properties (comparable to those of ketoconazole **1** against the two *Aspergillus* species) were shown by several sulfonates (**26–32** and **35**), by all the arylsulfonylcarbamyl derivatives (**36–40**), which together with the carbamyl/thiocarbamyl derivatives **41–47** were among the best antifungals in this series of compounds (MIC-s in the range of 1.2–4 μg/mL). Some esters containing sulfonamido moieties, such as **54, 55** and **56**, also showed effective antifungal properties (Table II). Thus, substitutions leading to good antifungal action included: sulfanilyl, 3-methoxysulfanilyl, pentafluoro-phenylsulfonyl/carboxyl, arylsulfonylcarbamyl, benzoylthiocarbamyl and arylcarbamyl. In this last subseries of derivatives, best activity was correlated with the presence of one or more halogen atoms in the arylcarbamyl moiety, (v) the susceptibility of the three fungi strains to this class of antifungals was generally the following: *A. flavus* > *A. niger* > *C. albicans*. It should also be noted that the new compounds were much less effective against the last pathogen, as compared to ketoconazole, but generally the most active derivatives showed the same potency as **1** against the two *Aspergilli*.

In order to investigate the possible mechanism of antifungal action of these new derivatives, supplementary experiments were performed. Thus, it is well known that ketoconazole **1** acts as an effective inhibitor of lanosterol 14-α-demethylase (CYP51A1),[1–5] a microsomal cytochrome P-450 dependent enzyme system belonging to a rapidly expanding gene superfamily involved in sterol biosynthesis in fungi, plants and animals.[26–30] CYP51A1 has been shown to catalyze the conversion of lanosterol to the 14-desmethylated derivative, ergosterol, through a complicated oxidative reaction sequence. Inhibition of this enzyme in fungi causes ergosterol depletion and accumulation of 14-methylsterols in the cell membranes, leading to serious impairment of the membrane function, which subsequently causes apoptosis and the death of the pathogens.[26–30]

In order to investigate whether the compounds reported here act as ergosterol biosynthesis inhibitors, similarly to the azole antifungals, the amounts of ergosterol present in *A. flavus* cultures after treatment with different concentrations of some of the new inhibitors of types **28, 38, 47,** and ketoconazole **1** (as standard) was determined by means of an HPLC method[16–20,25] (Table III).

The data of Table III show that at low concentrations of both types of inhibitor (ketoconazole, and dioxines, respectively), an amount of 85–92% of ergosterol is still synthesized, as compared to the corresponding amounts

TABLE III Levels of ergosterol in *A. flavus* cultures after treatment with different concentrations of the azole CYP51A1 inhibitor ketoconazole **1** and the new compounds **28, 38, 47**

Inhibitor	*Concentration* (μg/mL)	*% Ergosterol**
Ketoconazole	0.01	89 ± 5
Ketoconazole	0.02	66 ± 7
Ketoconazole	0.05	8 ± 4
28	0.01	92 ± 6
28	0.05	60 ± 5
28	0.15	10 ± 2
38	0.01	91 ± 4
38	0.10	56 ± 5
38	0.25	21 ± 3
38	0.75	8 ± 2
47	0.01	85 ± 5
47	0.05	33 ± 4
47	0.15	7 ± 1

* Mean ± standard deviation ($n = 3$); The amount of ergosterol present in the same amount of wet cells from the culture grown in the absence of inhibitor is taken as 100%.

of sterol formed in cultures in which inhibitors have not been added, and which were taken as 100%. By increasing the concentrations of inhibitors used in the experiments, the amounts of detected ergosterol decreased dose-dependently. A similar effect was observed for the CYP51A1 inhibitor ketoconazole **1** as well as for the new compounds **28, 38** and **47** synthesized in the present study. These data allow us to propose a similar mechanism of action for the two classes of antifungal compounds, i.e., the inhibition of lanosterol-14-α-demethylase, although it is not improbable that our compounds might interfere with other enzyme(s) involved in the ergosterol biosynthetic pathway. In order to be sure that the mechanism of antifungal action really involves interaction with CYP51A1, spectroscopic studies of this metalloenzyme with azole/dioxine inhibitors should be performed. Although CYP51A1 has been cloned in several fungi/yeasts,[28–31] the clones are unfortunately not available to us for the moment and such experiments could not be performed.

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