Laboratory note

The antifungal activity of sulfonylamido derivatives of 2-aminophenoxathiin and related compounds

Claudiu T. Supuran^a*, Andrea Scozzafava^a, Fabrizio Briganti^a, George Loloiu^b, Ovidiu Maior^b

^aLaboratorio di Chimica Inorganica e Bioinorganica, Università degli Studi di Firenze, Via Gino Capponi 7, 50121 Firenze, Italy ^bDepartamentul de Chimie Organica, Universitatea Bucuresti, Sos. Panduri 92, Bucuresti, Romania

(Received 16 January 1998; accepted 29 April 1998)

Abstract – Aryl/alkyl-sulfonylamido, arylsulfenylamido, arylcarboxamido and ureido/thioureido derivatives of 2-aminophenoxathiin were prepared by reaction of the title compound with sulfonyl/sulfenyl halides, sulfonic acid anhydrides, acyl chlorides, tosyl isocyanate, aryl/allyl isocyanates or isothiocyanates. Some of these derivatives, containing free amino groups, have been further derivatized by reaction with 2,4,6-trisubstituted-pyrylium salts, aryl/allyl isocyanate/isothiocyanates or tosyl isocyanate. Several of the newly synthesized compounds act as effective antifungal agents against *Aspergillus* and *Candida spp.*, some of them showing activities comparable to ketoconazole or itraconazole (against the aspergilli) but being much less effective against *Candida*. The mechanism of action of these compounds involves inhibition of ergosterol biosynthesis, and probably interaction with lanosterol-14- α -demethylase (CYP51A1), since reduced amounts of ergosterol were evidenced by means of HPLC in cultures of the sensitive strain *A. niger* treated with some of these inhibitors. Thus, the two classes of antifungal compounds, i.e. the azoles and the new derivatives reported here, might possess a similar mechanism of action at molecular level. © Elsevier, Paris

2-aminophenoxathiin / sulfonyl halides / iso(thio)cyanates / (thio)ureas / antifungal compounds / ergosterol biosynthesis inhibitors

1. Introduction

Polynuclear heterocyclic derivatives show a large variety of interesting biological activities, many widely used drugs belonging to this category of compounds. Thus, phenothiazines were found to have antihistaminic and antipsychotic action [1, 2], dibenzazepines and related derivatives are among the most widely used antidepressant and anxiolytic agents [1, 3], but antiarrhythmic [4], amoebicidic [5], fungistatic [6, 7], antibiotic/anticancer [8] action for derivatives belonging to the acridine, phenazine, phenothiazine or phenoxazone ring systems have also been reported [1–8].

On the other hand, phenoxathiin derivatives although structurally similar to the ring systems mentioned above, were much less investigated for their biological activity. It was only recently reported by this group the preparation of some enzyme inhibitors containing phenoxathiin-2-yl or 10,10-dioxa-phenoxathiin-2-yl moieties in their molecule [9, 10]. The interesting biological activity of the above-mentioned compounds prompted us to investigate novel derivatives from this class. 2-Aminophenoxathiin 1 constituted the starting material in the synthesis of pyridinium derivatives of type 2 previously reported [11], as well as for the synthesis of novel compounds of type 3-30, reported in the present paper (see *figure 1*).

In this paper we describe the preparation of sulfonamido derivatives of 2-aminophenoxathiin of type 3-30, obtained by reaction of 1 with sulfonyl halides or sulfonic acid anhydrides. Related compounds were prepared from 1 and sulfenyl chlorides, acyl chlorides, aryl/allyl isocyanates and isothiocyanates. Some of the above derivatives were further derivatized by means of reactions involving pyrylium salts, allyl/aryl/tosyl isocyanates or isothiocyanates. The new compounds reported here have been characterized by elemental analysis and standard spectroscopic methods (IR and ¹H-NMR spectroscopy) which confirmed their structure.

^{*}Correspondence and reprints

822



Figure 1. Structure of compounds 1, 2 and 3-30.

The new compounds reported in the present paper were designed in the search of novel classes of enzyme inhibitors with potentiality as antifungal drugs. Opportunistic fungal infections are an increasingly important cause of morbidity and mortality, with Aspergillus and Candida species being the most common such pathogens [12]. Members of the genus Aspergillus are associated with an impressive spectrum of diseases in humans, ranging from benign colonization of the lung to severe pathologies such as invasive aspergillosis or allergic bronchopulmonary aspergillosis [13]. Although A. fumigatus has been identified as the most common etiological agent in the human diseases, being considered a pathogen and allergen at the same time [13, 14], recent data showed the apparently benign A. niger and flavus to be involved in life-threatening conditions such as fungal endocarditis [15] as well as endogenous endophthalmitis, leading in many cases to an irreversible loss of visual outcome [16].

The mechanism of action of many fungistatic drugs, such as the widely clinically used azoles ketoconazole, itraconazole, etc. [17-20], consists in inhibition of sterol 14- α -demethylase (CYP51A1), a microsomal cytochrome P-450 dependent enzyme system belonging to a gene superfamily involved in sterol biosynthesis in fungi, plants and animals [21-23]. CYP51A1 has been shown to catalyze the conversion of lanosterol to the 14desmethylated derivative, ergosterol, through the oxidative sequence of alcohol, aldehyde, followed by decarboxylation and release of formic acid [24-26]. It appeared thus of interest to investigate whether the obtained derivatives 3-42 with potential antifungal activity might share a common mechanism of action with the previously mentioned azoles. Thus, we assayed the obtained compounds against several widespread fungi, such as Aspergillus and Candida spp., evidencing interesting activity for some of them. For the most active compound against A. niger, the amount of ergosterol after treatment with different concentrations of the new and azole inhibitors have been determined by means of HPLC, being thus shown that the antifungal effect of the new class of compounds is probably indeed due to inhibition of ergosterol biosynthesis. Whether CYP51A1 is indeed the inhibited enzyme, or whether other enzymes involved in the ergosterol pathway interact with our compounds, is for the moment an unresolved problem.

2. Chemistry

The new compounds **3–30**, prepared by reaction of 2-aminophenoxathiin 1 with sulfonyl halides, sulfonic acid anhydrides, acyl chlorides as well as isocyanates/iso-thiocyanates, are shown in *table I*. Generally they were synthesized from 1 and the corresponding sulfonyl chloride/fluoride in acetonitrile and in the presence of triethylamine. The only exceptions are constituted by the trifluoromethyl derivative 5, obtained from 1 and triflic anhydride, in acetone as solvent at molar ratios of the two reactants of 2:1, and the 2-carboxyphenyl derivatives **19** and **20**, respectively, prepared from 1 and sulfobenzoic acid cyclic anhydrides in refluxing acetonitrile.

Further derivatization of compounds 16 and 17, containing a free NH_2 group, with 2,4,6-trisubstitutedpyrylium perchlorates, tosyl isocyanate, 3,4-dichlorophenyl isocyanate and allyl isothiocyanate afforded the new compounds **31–42** (*figure 2*).

The new derivatives were characterized by elemental analysis ($\pm 0.4\%$ of the theoretical values, calculated for the proposed formulas, see the experimental part), IR and ¹H-NMR spectroscopy.

3. Pharmacology

Antifungal activity of the new compounds reported here has been determined by a modification of the growth method, as reported earlier by this group [27, 28]. The activity of the new compounds is shown in *table II*. Two *Aspergillus* species and one strain of *Candida albicans* were included in our assays, as these are widespread fungi, which easily develop resistance against many antifungal compounds [29]. Ketoconazole, a well-known imidazole possessing strong antifungal activity has been included as standard in these assays.

The amounts (percentuals) of sterols present in the culture prior and after treatment with different concentrations of inhibitors are shown in *table 111*, and were determined by means of a reverse-phase HPLC method [30, 31].



|--|

Compound	R	Yield	Synthesis method ^a
3	Me ₂ NSO ₂	54	Α
4	PhCH ₂ SO ₂	51	В
5	CF_3SO_2	60	С
6	$p-F-C_6H_4-SO_2$	62	Α
7	$p-Cl-C_6H_4-SO_2$	76	Α
8	p-Br–C ₆ H ₄ –SO ₂	73	Α
9	$p-I-C_6H_4-SO_2$	85	А
10	$p-CH_3-C_6H_4-SO_2$	69	Α
11	$p-O_2N-C_6H_4-SO_2$	55	Α
12	$m - O_2 N - C_6 H_4 - SO_2$	55	А
13	$o - O_2 N - C_6 H_4 - SO_2$	42	Α
14	$3-C_{1}-4-O_{2}N-C_{6}H_{3}-SO_{2}$	55	А
15	p-AcNH–C ₆ H ₄ –SO ₂	89	Α
16	$p-H_2N-C_6H_4-SO_2$	50	В
17	$m-H_2N-C_6H_4-SO_2$	39	В
18	$C_6F_5-SO_2$	82	Α
19	o-HOOC-C ₆ H ₄ -SO ₂	93	D
20	o-HOOC-C ₆ Br ₄ -SO ₂	88	D
21	$p-CH_3O-C_6H_4-SO_2$	69	Α
22	$2,4,6-(CH_3)_3-C_6H_2-SO_2$	69	А
23	Ph ₂ N-CO	39	А
24	isonicotinoyl	66	Α
25	2,4-Cl ₂ C ₆ H ₃ CO	58	А
26	$3,4-Cl_2C_6H_3NHCO$	91	E
27	p-Me-C ₆ H ₄ SO ₂ NHCO	95	Е
28	CH ₂ =CHCH ₂ NHCS	38	Е
29	$p - \overline{O_2} N - \overline{C_6} H_4 - S$	55	А
30	$o - O_2 N - C_6 H_4 - S$	42	А

^a A: 2-amino-phenoxathiine + RSO₂Cl (or RCOCl or RSCl); B: 2-amino-phenoxathiine + RSO₂F; C: 2-amino-phenoxathiine + triflic anhydride; D: 2-amino-phenoxathiine + sulfobenzoic cyclic anhydride; E: 2-amino-phenoxathiine + RNCO (or RNCS).

4. Discussion

Analytic and spectral data confirmed the proposed structure for the newly synthesized derivatives **3–42**. In the IR spectra of compounds **3–42**, the following bands were detected: (i) the intense sulfonamido vibrations, at 1140–1175 cm⁻¹ (SO₂^{sym}), and 1320–1345 cm⁻¹ (SO₂^{as}), respectively, for compounds **3–22**; (ii) the NH vibrations at around 3060 cm⁻¹; (iii) bands of the aromatic rings (C=C) around 1490–1500 cm⁻¹, as well as bands due to the other structural elements present in these molecules

(such as NH₂ for derivatives 16, 17 or COOH for 19 and 20); (iv) the strong amide vibrations, at 1680–1700 cm⁻¹ (amide I), 1520–1540 cm⁻¹ (amide II) and 1290–1300 cm⁻¹ (amide III), respectively, for the two carboxamides 24 and 25, as well as for ureas 26, 27 and 33–36. For the latter derivatives (ureas) the amide I bands appeared at 1730 cm⁻¹.

In the 200 MHz ¹H-NMR spectra of derivatives **3–30** (in DMSO- d_6), the following signals were detected: (i) the SO₂NH protons as a broad singlet at 8.05–8.35 ppm, in fast exchange with the bulk solvent, and the signal



41: m-isomer X = Y = Ph **42:** p-isomer, X = Y = Ph

Figure 2.

disappears by addition of D_2O into the NMR tube after 5–10 min; (ii) the seven aromatic protons of the phenoxathiin-2-yl moiety as a multiplet in the range 7.50–7.95 ppm; (iii) the aromatic protons of the substituted-phenyl moiety directly bound to the heterocyclic ring appeared as an AA'BB' multiplet for the *p*-substituted derivatives, whereas the signals of the protons of the other R groups of compounds **3–30** appeared in their normal ranges (see Experimental protocols for details). For derivatives **31–42**, the spectral data also confirmed the proposed structures (see Experimental protocols).

The new compounds **3–42** described here, represent a novel class of antifungal derivatives, with broad activity against fungi and moulds such as *Aspergillus* and *Can-dida spp. (table II)*.

As seen from thee data of *table II*, the aminophenoxathiin **1** is completely inactive as antifungal com-

pound, against all three investigated organisms, whereas sulfonamido, carboxamido, sulfenamido its and urea/thiourea derivatives exhibit in different degrees such an action. In the series of synthesized derivatives 3-30, best activity was correlated with the presence of nitro moieties in the arylsulfonamido/sulfenamido groups linked to the heterocyclic ring (compounds 12-14, 29 and 30), pentafluorophenylsulfonamido (18), or diphenylcarbamoylamido (23) moieties. Monohalogeno atoms or other groups such as amino, alkyls, etc., were less effective in inducing strong antifungal activity in these compounds, but the dichloro-substituted compounds 25 and especially 26 showed good activities. Appreciable inhibitory activity was detected for the compounds obtained by derivatization of the amino compounds 16 and 17. Thus, thioureas 31 and 32, as well as ureas 33-36 were among the most active compounds in the whole series, with potencies superior to ketoconazole against

Compound	MIC (µg/mL)			
	A. flavus C1150	A. niger C418	Candida albicans C316	
1	> 125	> 125	> 125	
3	20	12	10	
4	15	10	17	
5	27	51	33	
6	8	9	8	
7	11	7	9	
8	15	10	8	
9	15	12	10	
10	12	8	9	
11	6	7	4	
12	4	5	5	
13	5	2	3	
14	2	2	1	
15	8	10	7	
16	12	13	16	
17	15	8	10	
18	2	1	0.5	
19	45	36	31	
20	23	10	16	
21	5	7	6	
22	5	5	5	
23	1	2	0.4	
24	11		12	
25	2	4	5	
26	06	0.9	0.8	
20	8	15	12	
28	7	10	5	
20	07	1.2	5	
30	0.4	0.8	1.1	
30	0.8	1.5	1.4	
32	1.2	1.5	1.4	
32	0.4	0.8	0.5	
34	0.9	0.0	0.5	
35	0.9	0.7	0.0	
35	0.4	0.7	0.5	
27	24	0.7	0.0	
28	24	15	13	
30	10	1.0	14	
37 /0	14	10	/ 8	
4U 1	14	9 0.2	0	
41	1.0	0.3	3	
44 Vete e en en el e	1.0	U.4	3	
Itaanarala	1.2	1.8	0.00	
	0.9	0.2	0.02	

Table II. Antifungal activity of compounds 1-42 against several organisms.

the two Aspergillus, but were less effective against Candida, as compared to this drug. In the case of the pyridinium salts, 2,4,6-trisubstituted pyridinium derivatives **37** and **38** were moderately active, but introducing an increasing number of phenyl moieties instead of the methyl ones led to improved activity, with the 2,4,6-

triphenylpyridinium salts **41** and **42** again more effective than ketoconazole against the two *Aspergillus* species. Probably the ineffectiveness of the trimethylpyridinium derivatives **37** and **38** might be accounted to their very low lipophilicity and impossibility to penetrate through biological membranes in vivo [32]. For compounds

Table III. Levels of ergosterol in *A. niger* cultures after treatment with different concentrations of the azole CYP51A1 inhibitor itraconazole and compounds **35** and **41**.

Inhibitor	Concentration (µg/mL)	% Ergosterol ^a
Itraconazole	0.01	78 ± 5
Itraconazole	0.05	41 ± 7
Itraconazole	0.10	11 ± 4
35	0.01	96 ± 3
35	0.10	64 ± 7
35	0.25	23 ± 6
35	0.60	9 ± 3
41	0.01	87 ± 7
41	0.05	35 ± 4
41	0.10	12 ± 5

^a Mean \pm 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%.

31-42 one should also note that generally the *meta*-substituted isomers were more active than the *para*-substituted ones.

A verification of our initial hypothesis that the compounds reported here act as ergosterol biosynthesis inhibitors has been provided by data shown in table III. The amounts of ergosterol present in A. niger cultures after treatment with different concentrations of inhibitors (itraconazole, a potent CYP51A1 inhibitor [20-23] has also been included in the study, since ketoconazole had a weak inhibitory effect against aspergilli) proved that at low concentrations of inhibitor, around 80-96% of ergosterol (as compared to the amount of sterol formed in cultures in which inhibitors have not been added, and which was considered 100%) is still synthesized. By increasing the concentrations of inhibitors used in the experiments, the amount of synthesized ergosterol decreased dosedependently. A similar effect has been observed for the well-known CYP51A1 inhibitor itraconazole as well as for the compounds **35** and **41** 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 (see figure 3).

The new class of inhibitors of the type described by us here probably meet structural requirements enabling them to strongly bind to one or more of the enzymes involved in the ergosterol biosynthesis, and inhibit in this way the whole steroid synthesis. Whether this enzyme is indeed CYP51A1 or another enzyme involved in this biosynthetic pathway cannot be precisely stated at the moment.

5. Experimental protocols

5.1. Chemistry

Melting points were obtained with a heating plate microscope and are uncorrected. IR spectra were recorded in CsBr pellets with a Nicolet 2DXFT-IR apparatus. ¹H-NMR spectra were recorded with a Bruker CPX 200 instrument operating at 200 MHz. Elemental analysis was done by combustion with a Carlo Erba Instrument.

2-Aminophenoxathiin was prepared by literature procedures [37]. Ketoconazole and itraconazole were from Sigma, whereas sulfonyl halides, sulfonic acid anhydrides, tosyl isocyanate, triethylamine, allyl isothiocyanate, 3,4-dichorophenyl isocyanate, and acyl halides were commercially available from Acros, E. Merck or Aldrich, and were used without further purification. 2,4,6-Trimethyl-, 2,4,6-tripheyl- and 2,6-dimethyl-4-phenyl-pyrylium perchlorates were prepared by literature procedures [38]. Ergosterol and lanosterol used as standards in the HPLC measurements were from Sigma.

5.2. Synthesis of derivatives 3-30

Methods A and B: 108 mg (5 mmol) of 2-aminophenoxathiin suspended in 10 mL of acetonitrile were treated with 5 mmol of sulfonyl/sulfenyl chloride (method A) or fluoride (method B)



44: ergosterol

Figure 3. Structure of compounds 43 and 44.

dissolved in a small amount of anhydrous acetonitrile. The stoichiometric amount of triethylamine was added, and the mixture was stirred at 40 °C for 4 h (A) or at 60 °C for 6 h (B), then the solvent was evaporated in vacuo and the reaction mixture poured into 40 mL of water and ice. The precipitated sulfonylamido derivatives were recrystallized from ethanol-water (1:1, v/v).

Method C: 215 mg (10 mmol) of 2-aminophenoxathiine and 0.84 mL (5 mmol) of triffic anhydride were suspended in 10 mL of acetone and magnetically stirred at 4 $^{\circ}$ C for 15 h. The solvent was then evaporated in vacuo, and the tan residue treated with 10 mL of cold water. The triffate salt of 2-aminophenoxathiine being water soluble has thus been separated from 5 by filtration. The latter compound was recrystallized from ethanol.

Method D: 108 mg (5 mmol) of 2-aminophenoxathiine and 5 mmol of sulfobenzoic cyclic anhydride or tetrabromo-O-sulfobenzoic cyclic anhydride were heated at refluxation in 50 mL of anhydrous acetonitrile for 2 h, with a small amount of p-toluenesulfonic acid added as catalyst. After evaporation of the solvent, the products **19**, **20** were recrystallized from ethanol.

Method E: 108 mg (5 mmol) of 2-aminophenoxathiine and 10 mmol of isocyanate or isothiocyanate were heated at refluxation in 50 mL of anhydrous acetonitrile for 2-10 h, with a small amount (0.2 mL) of triethylamine added as catalyst. After evaporation of the solvent, the crude products were recrystallized from ethanol or methanol.

5.3. Synthesis of derivatives 31-34

2 mMol of amino-derivative **16** or **17** dissolved in 15 mL of anhydrous acetonitrile were heated at reflux and 0.47 g (2.5 mmol) of 3,4-dichlorophenyl isocyanate (or the equivalent amount of allyl isothiocyanate) dissolved in 5 mL of the same solvent were added dropwise. The mixture was heated at reflux for 3 h, then part of the solvent was evaporated and the obtained mixture was left at 0 °C overnight. The precipitated derivatives were filtered and recrystal-lized from dioxane. Yields were around 80-90%.

5.4. Synthesis of derivatives 35 and 36

2 mMol of amino-derivative 16 or 17 dissolved in 20 mL of anhydrous acetonitrile were treated with 0.30 mL (2 mmol) tosyl isocyanate. The mixture was stirred at room temperature for 1 h, and then the precipitated derivatives were filtered off and recrystallized from ethanol. Yields were over 95%.

5.5. Synthesis of derivatives 37-42

2 mMol of amino-derivative 16 or 17 dissolved in 30 mL of anhydrous ethanol were treated with the stoichiometric amount of 2,4,6-trisubstitutedpyrylium perchlorate dissolved in the minimum amount of the same solvent. The mixture was magnetically stirred at room temperature for 15 min, then 0.27 mL (2 mmol) of triethylamine were added and stirring was continued for another 2 h. After this time, 1.5 mL of acetic acid were added and the reaction mixture was heated at reflux for 3 h. After cooling, the pyridinium salt was precipitated by addition of 100 mL of diethyl ether. Filtration and recrystallization from *iso*-propanol afforded the title compounds with yields of 55–58%.

5.5.1. 2-(N,N-Dimethylsulfamoylamido)-phenoxathiin 3: tan crystals, m.p. 119–122 °C. IR (KBr), cm⁻¹: 1140 (SO₂^{sym}), 1339

5.5.2. 2-Phenylmethylsulfonylamido-phenoxathiin 4: tan crystals, m.p. 160–161 °C. IR (KBr), cm⁻¹: 1176 (SO_2^{sym}), 1360 (SO_2^{as}), 3060 (NH); ¹H-NMR (DMSO-d₆), δ , ppm: 3.17 (s, 2H, PhCH₂); 7.12–7.49 (m, 5H, ArH from Ph); 7.58–7.93 (m, 7H, ArH from phenoxathiin); 8.11 (s, 1H, SO₂NH); Anal. ($C_{19}H_{15}NO_3S_2$) C, H, N.

5.5.3. 2-Trifluoromethylsulfonylamidophenoxathiin 5: colorless crystals, m.p.152–153 °C. IR (KBr), cm⁻¹: 1169 (SO_2^{sym}), 1351 (SO_2^{as}), 3060 (NH); ¹H-NMR (DMSO- d_6), δ , ppm: 7.50–7.93 (m, 7H, ArH); 8.35 (s, 1H, SO₂NH); Anal. ($C_{13}H_8F_3NO_3S_2$) C, H, N.

5.5.4. 2-(4-Fluorophenylsulfonylamido)-phenoxathiin **6**: color-less crystals, m.p. 194–195 °C. IR (KBr), cm⁻¹: 1171 (SO₂^{sym}), 1366 (SO₂^{as}), 3060 (NH); ¹H-NMR (DMSO-d₆), δ , ppm: 7.11–7.43 (m, AA'BB', J_{AB} = 7.4 Hz, 4H, ArH, *p*-F-phenylene); 7.55–7.94 (m, 7H, ArH, from phenoxathiin); 8.05 (s, 1H, SO₂NH); Anal. (C₁₈H₁₂FNO₃S₂) C, H, N.

5.5.5. 2-(4-Chlorophenylsulfonylamido)-phenoxathiin 7: colorless crystals, m.p. 205–207 °C. IR (KBr), cm⁻¹: 1175 (SO₂^{ssym}), 1367 (SO₂^{us}), 3065 (NH); ¹H-NMR (DMSO-d₆), δ , ppm: 7.10–7.43 (m, AA'BB', J_{AB} = 7.4 Hz, 4H, ArH, *p*-Cl-phenylene); 7.50–7.94 (m, 7H, ArH from phenoxathiin); 8.06 (s, 1H, SO₂NH); Anal. (C₁₈H₁₂CINO₃S₂) C, H, N.

5.5.6. 2-(4-Bromophenylsulfonylamido)-phenoxathiin 8: colorless crystals, m.p. 213–215 °C. IR (KBr), cm⁻¹: 1179 (SO₂^{sym}), 1376 (SO₂^{as}), 3065 (NH); ¹H-NMR (DMSO-d₆), δ , ppm: 7.15–7.52 (m, AA'BB', J_{AB} = 7.4 Hz, 4H, ArH, *p*-Br-phenylene); 7.55–7.98 (m, 7H, ArH, from phenoxathiin); 8.05 (s, 1H, SO₂NH); Anal. (C₁₈H₁₂BrNO₃S₂) C, H, N.

5.5.7. 2-(4-Iodophenylsulfonylamido)-phenoxathiin **9**: colorless crystals, m.p. 220–221 °C. IR (KBr), cm⁻¹: 1185 (SO₂^{sym}), 1380 (SO₂^{as}), 3060 (NH); ¹H-NMR (DMSO-*d*₆), δ, ppm: 7.17–7.48 (m, AA'BB', J_{AB} = 7.4 Hz, 4H, ArH, *p*-I-phenylene); 7.54–7.96 (m, 7H, ArH, from phenoxathiin); 8.09 (s, 1H, SO₂NH); Anal. (C₁₈H₁₂INO₃S₂) C, H, N.

5.5.8. 2-*p*-Tosylamidophenoxathiin **10**: tan crystals, m.p. 182–184 °C. IR (KBr), cm⁻¹: 1165 (SO₂^{sym}), 1350 (SO₂^{as}), 3060 (NH); ¹H-NMR (DMSO- d_6), δ, ppm: 2.50 (s, 3H, Me from tosyl); 7.05–7.46 (m, AA'BB', $J_{AB} = 7.4$ Hz, 4H, ArH, *p*-Me-phenylene); 7.52–7.89 (m, 7H, ArH, from phenoxathiin); 8.05 (s, 1H, SO₂NH); Anal. (C₁₉H₁₅NO₃S₂) C, H, N.

5.5.9. 2-(4-Nitrophenylsulfonylamido)-phenoxathiin 11: yellow crystals, m.p. 210–212 °C. IR (KBr), cm⁻¹: 1150 (SO₂^{sym}), 1366 (SO₂^{as}), 3060 (NH); ¹H-NMR (DMSO- d_6), δ, ppm: 7.08–7.49 (m, AA'BE', J_{AB} = 7.4 Hz, 4H, ArH, p-O₂N-phenylene); 7.53–7.90 (m, 7H, ArH, from phenoxathiin); 8.10 (s, 1H, SO₂NH); Anal. (C₁₈H₁₂N₂O₅S₂) C, H, N.

5.5.10. 2-(3-Nitrophenylsulfonylamido)-phenoxathiin 12: yellow crystals, m.p. 208–210 °C. IR (KBr), cm⁻¹: 1152 (SO₂^{sym}),

1374 (SO₂^{as}), 3065 (NH); ¹H-NMR (DMSO- d_6), δ , ppm: 7.08–7.50 (m, 4H, ArH, *m*-O₂N-phenylene); 7.53–7.89 (m, 7H, ArH, from phenoxathiin); 8.10 (s, 1H, SO₂NH); Anal. (C₁₈H₁₂N₂O₅S₂) C, H, N.

5.5.11. 2-(2-Nitrophenylsulfonylamido)-phenoxathiin 13: yellow crystals, m.p. 200-201 °C. IR (KBr), cm⁻¹: 1152 (SO₂^{sym}), 1369 (SO₂^{as}), 3060 (NH); ¹H-NMR (DMSO- d_6), δ , ppm: 7.02-7.49 (m, 4H, ArH, o-O₂N-phenylene); 7.55-7.97 (m, 7H, ArH, from phenoxathiin); 8.07 (s, 1H, SO₂NH); Anal. (C₁₈H₁₂N₂O₅S₂) C, H, N.

5.5.12. 2-(3-Chloro-4-nitrophenylsulfonylamido)-phenoxathiin 14: yellow crystals, m.p. 189–191 °C. IR (KBr), cm⁻¹: 1157 (SO₂^{sym}), 1369 (SO₂^{as}), 3060 (NH); ¹H-NMR (DMSO-d₆), δ , ppm: 7.08–7.67 (m, 3H, ArH, 3-Cl-4-O₂N-phenyl); 7.56–7.98 (m, 7H, ArH, from phenoxathiin); 8.11 (s, 1H, SO₂NH); Anal. (C₁₈H₁₁ClN₂O₅S₂) C, H, N.

5.5.13. 2-(4-Acetylaminophenylsulfonylamido)-phenoxathiin 15: colorless crystals, m.p. 217–219 °C. IR (KBr), cm⁻¹: 1155 (SO₂^{sym}), 1296 (amide III), 1350 (SO₂^{as}), 1533 (amide II); 1680 (amide I); 3066 (NH); ¹H-NMR (DMSO- d_6), δ , ppm: 1.80 (s, 3H, Me from Ac); 7.07–7.46 (m, AA'BB', $J_{AB} = 7.4$ Hz, 4H, ArH, *p*-AcNH-phenylene); 7.53–7.96 (m, 7H, ArH, from phenoxathiin); 8.08 (s, 1H, SO₂NH); Anal. (C₂₀H₁₆N₂O₄S₂) C, H, N.

5.5.14. 2-(4-Aminophenylsulfonylamido)-phenoxathiin 16: colorless crystals, m.p. 230–232 °C. IR (KBr), cm⁻¹: 1155 (SO₂^{sym}), 1347 (SO₂^{as}), 3060 (NH); ¹H-NMR (DMSO-*d*₆), δ , ppm: 5.42 (s, 2H, *H*₂N-phenylene); 7.05–7.50 (m, AA'BB', *J*_{AB} = 7.3 Hz, 4H, ArH, *p*-H₂N-phenylene); 7.54–7.90 (m, 7H, ArH, from phenoxathiin); 8.11 (s, 1H, SO₂NH); Anal. (C₁₈H₁₄N₂O₃S₂) C, H, N.

5.5.15. 2-(3-Aminophenylsulfonylamido)-phenoxathiine 17: tan crystals, m.p. 220–222 °C. IR (KBr), cm⁻¹: 1172 (SO₂^{sym}), 1364 (SO₂^{as}), 3060 (NH); ¹H-NMR (DMSO- d_6), δ , ppm: 5.11 (s, 2H, H_2 N-phenylene) 7.21–7.45 (m, 4H, ArH, *m*-H₂N-phenylene); 7.54–7.90 (m, 7H, ArH, from phenoxathiin); 8.05 (s, 1H, SO₂NH); Anal. (C₁₈H₁₄N₂O₃S₂) C, H, N.

5.5.16. 2-(Pentafluorolphenylsulfonylamido-phenoxathin 18: tan crystals, m.p. 157–158 °C (dec). IR (KBr), cm⁻¹: 1148 (SO₂^{sym}), 1330 (SO₂^{as}), 3060 (NH); ¹H-NMR (DMSO-d₆), δ , ppm: 7.54–7.97 (m, 7H, ArH, from phenoxathiin); 8.38 (s, 1H, SO₂NH); Anal. (C₁₈H₈F₅NO₃S₂) C, H, N.

5.5.17. 2-(2-Carboxyphenylsulfonylamido)-phenoxathiine **19**: tan crystals, m.p. 201–202 °C. IR (KBr), cm⁻¹: 1153 (SO₂^{sym}), 1355 (SO₂^{as}), 1720 (COOH); 3065 (NH); ¹H-NMR (DMSO-d₆), δ , ppm: 7.15–7.43 (m, 4H, ArH, o-HOOC-phenylene); 7.50–7.90 (m, 7H, ArH, from phenoxathiin); 8.05 (s, 1H, SO₂NH); 10.15 (br s, 1H, COOH); Anal. (C₁₉H₁₃NO₅S₂) C, H, N.

5.5.18. 2-(2-Carboxytetrabromophenylsulfonylamido)-phenoxathiine **20**: tan crystals, m.p. 181–182 °C (dec). IR (KBr), cm⁻¹: 1158 (SO₂^{sym}), 1371 (SO₂^{as}), 1720 (COOH); 3060 (NH); ¹H-NMR (DMSO- d_6), δ, ppm: 7.50–7.90 (m, 7H, ArH, from phenoxathiin); 8.09 (s, 1H, SO₂NH); 10.40 (br s, 1H, COOH); Anal. (C₁₉H₉Br₄NO₅S₂) C, H, N. 5.5.19. 2-(4-Methoxyphenylsulfonylamido)-phenoxathiin 21: white crystals, m.p. 180–181 °C. IR (KBr), cm⁻¹: 1167 (SO₂^{sym}), 1350 (SO₂^{as}), 3060 (NH); ¹H-NMR (DMSO-d₆), δ, ppm: 3.50 (s, 3H, Me); 7.05–7.48 (m, AA'BB', J_{AB} = 7.4 Hz, 4H, ArH, *p*-MeOphenylene); 7.52–7.91 (m, 7H, ArH, from phenoxathiin); 8.09 (s, 1H, SO₂NH); Anal. (C₁₉H₁₅NO₄S₂) C, H, N.

5.5.20. 2-(2,4,6-Trimethylphenylsulfonylamido)-phenoxathiin 22: tan crystals, m.p. 158–160 °C. IR (KBr), cm⁻¹: 1165 (SO₂^{sym}), 1355 (SO₂^{as}), 3065 (NH); ¹H-NMR (DMSO-d₆), δ , ppm: 2.50 (s, 3H, 4-Me); 2.71 (s, 6H, 2,6-Me₂); 7.35 (s, 2H, ArH, 3,5-H from mesityl); 7.52–7.89 (m, 7H, ArH, from phenoxathiin); 8.05 (s, 1H, SO₂NH); Anal. (C₂₁H₁₉NO₃S₂) C, H, N.

5.5.21. 2-(N,N-Diphenylcarbamoylamido)-phenoxathiin 23: white crystals, m.p. 193–194 °C. IR (KBr), cm⁻¹: 1295 (amide III); 1520 (amide II); 1680 (amide I); 3060 (NH); ¹H-NMR (DMSO d_6), δ , ppm: 6.61 (br s, 1H, CONH); 7.18–7.43 (m, 10H, ArH from 2 Ph); 7.58–7.96 (m, 7H, ArH from phenoxathiin); Anal. (C₂₅H₁₈N₂O₂S) C, H, N.

5.5.22. 2-(Isonicotinoylamido)-phenoxathiin **24**: white crystals, m.p. 149–151 °C. IR (KBr), cm⁻¹: 1290 (amide III); 1540 (amide II); 1680 (amide I); 3060 (NH); ¹H-NMR (DMSO- d_6), δ, ppm: 7.15–7.72 (m, AA'BB', J_{AB} = 7.9 Hz, 4H, ArH); 7.58–7.96 (m, 7H, ArH from phenoxathiin); 7.98 (s, 1H, CONH); Anal. (C₁₈H₁₂N₂O₂S) C, H, N.

5.5.23. 2-(2,4-Dichlorophenylcarboxamido)-phenoxathiin **25**: white crystals, m.p. 174–175 °C. IR (KBr), cm⁻¹: 1300 (amide III); 1540 (amide II); 1700 (amide I); 3060 (NH); ¹H-NMR (DMSO d_6), δ, ppm: 7.05–7.64 (m, 3H, ArH); 7.90 (s, 1H, CONH); 7.58–7.96 (m, 7H, ArH from phenoxathiin); Anal. (C₁₉H₁₁Cl₂NO₂S) C, H, N.

5.5.24. 2-(3,4-Dichlorophenylureido)-phenoxathiin **26**: white crystals, m.p. 217–219 °C. IR (KBr), cm⁻¹: 1290 (amide III), 1550 (amide II); 1730 (amide I); 3060 (NH); ¹H-NMR (DMSO- d_6), δ , ppm: 5.23 (s, 2H, HN–CO–NH); 7.25–7.39 (m, 3H, ArH, dichlorophenyl); 7.58–7.96 (m, 7H, ArH from phenoxathiin); Anal. (C₁₉H₁₂Cl₂N₂O₂S) C, H, Cl, N.

5.5.25. 2-[4-(Tosylsulfonylureido)]phenoxathiin 27: colorless crystals, m.p. 228–229 °C. IR (KBr), cm⁻¹: 1150 (SO₂^{sym}), 1290 (amide III), 1360 (SO₂^{as}), 1566 (amide II); 1730 (amide I); 3060 (NH); ¹H-NMR (DMSO-*d*₆), δ, ppm: 2.50 (s, 3H, Me from tosyl); 5.20 (s, 2H, HN–CO–NH); 7.05–7.41 (m, AA'BB', J_{AB} = 7.1 Hz, 4H, ArH, phenylene from tosyl); 7.58–7.96 (m, 7H, ArH from phenoxathiin); Anal. (C₂₀H₁₆N₂O₄S₂) C, H, N.

5.5.26. N¹-(Phenoxathiin-2-yl)-N³-allyl-thiourea **28**: white crystals, m.p. 189–191 °C. IR (KBr), cm⁻¹: 1040 (thioamide III), 1547 (thioamide I), 3298 (NHCSNH); ¹H-NMR (DMSO- d_6), δ , ppm: 4.45–4.60 (m, 2H, CSNHC H_2); 5.60–5.97 (m, 3H, CH=CH₂); 6.70 and 6.82 (br s, 2H, NHCSNH); 7.58–7.96 (m, 7H, ArH from phenoxathiin); Anal (C₁₆H₁₄N₂OS₂) C, H, N.

5.5.27. 2-(4-Nitrobenzenesulfenylamido)-phenoxathiin **29**: yellow crystals, m.p. 210–213 °C, IR (KBr), cm⁻¹: 1075 and 1250 (NO₂), 1490, 1585 (C=C); 3230 (NH); ¹H-NMR (DMSO- d_6), δ ,

ppm: 5.12 (br s, 1H, NH); 7.15–7.43 (m, AA'BB', 4H, ArH from nitro-phenylene); 7.58–7.96 (m, 7H, ArH from phenoxathiin); Anal. ($C_{18}H_{12}N_2O_3S_2$) C, H, N.

5.5.28. 4-(2-Nitrobenzenesulfenylamido)-phenoxathiin **30**: pale yellow crystals, m.p. 175–177 °C, IR (KBr), cm⁻¹: 1080 and 1250 (NO₂), 1490, 1585 (C=C); 3260 (NH); ¹H-NMR (DMSO-*d*₆), δ , ppm: 5.19 (br s, 1H, NH); 7.29–7.53 (m, 4H, Ar H from *ortho*-substituted phenyl); 7.58–7.96 (m, 7H, ArH from phenoxathiin); Anal. (C₁₈H₁₂N₂O₃S₂) C, H, N.

5.5.29. N^{7} -(*Phenoxathiin-2-yl-aminosulfamoyl-phen-3-yl)-N*³allyl-thiourea 31: white crystals, m.p. 227–229 °C. IR (KBr), cm⁻¹: 1040 (thioamide III), 1170 (SO₂^{sym}), 1369 (SO₂^{as}), 1540 (thioamide I), 3295 (NHCSNH), 3360 (NH); ¹H-NMR (DMSO-*d*₆), δ , ppm: 4.40–4.60 (m, 2H, CSNH*CH*₂); 5.60–5.97 (m, 3H, CH=CH₂); 6.70 and 6.84 (br s, 2H, NHCSNH); 7.28–7.96 (m, 12H, ArH from phenoxathiin and 1,3-phenylene); Anal. (C₂₂H₁₉N₃O₃S₃) C, H, N.

5.5.30. N^{1} -(Phenoxathiin-2-yl-aminosulfamoyl-phen-4-yl)- N^{3} allyl-thiourea **32**: white crystals, m.p. 223–224 °C. IR (KBr), cm⁻¹: 1040 (thioamide III), 1177 (SO₂^{sym}), 1362 (SO₂^{as}), 1540 (thioamide I), 3295 (NHCSNH), 3360 (NH); ¹H-NMR (DMSO- d_{6}), δ , ppm: 4.50–4.63 (m, 2H, CSNHC H_{2}); 5.60–5.93 (m, 3H, CH=CH₂); 6.77 and 6.89 (br s, 2H, NHCSNH); 7.05–7.46 (m, AA'BB', J_{AB} = 7.1 Hz, 4H, ArH, p-substituted phenylene); 7.54–7.96 (m, 7H, ArH from phenoxathiin); Anal. (C₂₂H₁₉N₃O₃S₃) C, H, N.

5.5.31. N¹-(Phenoxathiin-2-yl-aminosulfamoyl-phen-3-yl)-N³-(3,4-dichlorophenyl)-urea **33**: colorless crystals, m.p. 185–186 °C. IR (KBr), cm⁻¹: 1165 (SO₂^{sym}), 1290 (amide III), 1367 (SO₂^{as}), 1540 (amide II); 1730 (amide I); 3060 (NH); ¹H-NMR (DMSOd₆), δ, ppm: 5.23 (br s, 2H, HN–CO–NH); 7.12–7.45 (m, 7H, ArH, from 1,3-phenylene + 3,4-dichlorophenyl); 7.54–7.97 (m, 7H, ArH from phenoxathiin); 8.08 (s, 1H, SO₂NH); Anal. (C₂₅H₁₇Cl₂N₃O₄S₂) C, H, Cl, N.

5.5.32. N^{\prime} -(Phenoxathiin-2-yl-aminosulfamoyl-phen-4-yl)- N^{3} -(3,4-dichlorophenyl)-urea **34**: colorless crystals, m.p. 213–215 °C. IR (KBr), cm⁻¹: 1171 (SO₂^{ssym}), 1295 (amide III), 1374 (SO₂^{as}), 1550 (amide II); 1730 (amide I); 3060 (NH); ¹H-NMR (DMSOd₆), δ, ppm: 5.20 (br s, 2H, HN–CO–NH); 7.02–7.49 (m, 7H, ArH, from 1,4-phenylene + 3,4-dichlorophenyl); 7.58–7.99 (m, 7H, ArH from phenoxathiin); 8.12 (s, 1H, SO₂NH); Anal. (C_{2s}H₁₇Cl₂N₃O₄S₂) C, H, Cl, N.

5.5.33. N^{7} -(Phenoxathiin-2-yl-aminosulfamoyl-phen-3-yl)- N^{3} -[4-(tosylsulfonyl])-urea 35: colorless crystals, m.p. 208–209 °C. IR (KBr), cm⁻¹: 1150 (SO₂^{sym}), 1290 (amide III), 1360 (SO₂^{as}), 1550 (amide II); 1730 (amide I); 3060 (NH); ¹H-NMR (DMSO-d₆), δ , ppm: 2.50 (s, 3H, Me from tosyl); 5.20 (br s, 2H, HN–CO–NH); 7.05–7.49 (m, 8H, ArH, 1,4-phenylene from tosyl + 1,3-phenylene); 7.53–7.92 (m, 7H, ArH from phenoxathiin); 8.06 (s, 1H, SO₂NH); Anal. (C₂₆H₂₁N₃O₆S₃) C, H, N.

5.5.34. N^{1} -(Phenoxathiin-2-yl-aminosulfamoyl-phen-4-yl)- N^{3} -[4-(tosylsulfonyl])-urea **36**: colorless crystals, m.p. 213–214 °C. IR (KBr), cm⁻¹: 1157 (SO₂^{sym}), 1285 (amide III), 1369 (SO₂^{as}), 1546

(amide II); 1730 (amide I); 3060 (NH); ¹H-NMR (DMSO- d_6), δ , ppm: 2.50 (s, 3H, Me from tosyl); 5.24 (br s, 2H, HN–CO–NH); 7.05–7.40 (m, 8H, ArH, 1,4-phenylene from tosyl + 1,4-phenylene from the arylsulfonamido ring); 7.51–7.95 (m, 7H, ArH from phenoxathiin); 8.13 (s, 1H, SO₂NH); Anal. (C₂₆H₂₁N₃O₆S₃) C, H, N.

5.5.35. 1-N-(Phenoxathiin-2-yl-aminosulfamoyl-phen-3-yl)-2,4,6-trimethylpyridinium perchlorate **37**: colorless crystals, m.p. 269–270 °C. IR (KBr), cm⁻¹: 1100 (perchlorate); 1155 (SO₂^{sym}), 1375 (SO₂^{as}), 1580 (C=C, C=N); 3060 (NH); ¹H-NMR (DMSOd₆), δ , ppm: 2.45 (s, 6H, 2,6-Me₂); 2.70 (s, 3H, 4-Me); 7.09–7.48 (m, 4H, ArH, 1,3-phenylene bound to pyridinium); 7.55 (s, 2H, ArH, 3,5-H from pyridinium); 7.51–7.95 (m, 7H, ArH from phenoxathiin); 8.08 (s, 1H, SO₂NH); Anal. (C₂₆H₂₂N₂O₃S₂+ClO₄⁻) C, H, N.

5.5.36. *I-N-(Phenoxathiin-2-yl-aminosulfamoyl-phen-4-yl)*-2,4,6-trimethylpyridinium perchlorate **38**: colorless crystals, m.p. 293–294 °C (dec). IR (KBr), cm⁻¹: 1100 (perchlorate); 1158 (SO₂^{sym}), 1362 (SO₂^{as}), 1595 (C=C, C=N); 3060 (NH); ¹H-NMR (DMSO-*d*₆), δ, ppm: 2.47 (s, 6H, 2,6-Me₂); 2.70 (s, 3H, 4-Me); 7.09–7.48 (m, AA'BB', *J*_{AB} = 7.3 Hz, 4H, ArH, 1,4-phenylene bound to pyridinium); 7.60 (s, 2H, ArH, 3,5-H from pyridinium); 7.50–7.95 (m, 7H, ArH from phenoxathiin); 8.05 (s, 1H, SO₂NH); Anal. (C₂₆H₂₂N₂O₃S₂+ClO₄⁻) C, H, N.

5.5.37. 1-N-(Phenoxathiin-2-yl-aminosulfamoyl-phen-3-yl)-2,6dimethyl-4-phenyl-pyridinium perchlorate **39**: tan crystals, m.p. 275–277 °C. IR (KBr), cm⁻¹: 1100 (perchlorate); 1155 (SO₂^{sym}), 1375 (SO₂^{as}), 1580 (C=C, C=N); 3060 (NH); ¹H-NMR (DMSOd₆), δ, ppm: 2.47 (s, 6H, 2,6-Me₂); 7.09–7.52 (m, 9H, ArH, 1,3-phenylene bound to pyridinium + 4-Ph); 7.51–7.90 (m, 7H, ArH from phenoxathiin); 7.95 (s, 2H, ArH, 3,5-H from pyridinium); 8.13 (s, 1H, SO₂NH); Anal. (C₃₆H₂₆N₂O₃S₂⁺ClO₄⁻) C, H, N.

5.5.38. *1-N-(Phenoxathiin-2-yl-aminosulfamoyl-phen-4-yl)-2,6dimethyl-4-phenyl-pyridinium perchlorate* **40**: colorless crystals, m.p. 280--283 °C (dec). IR (KBr), cm⁻¹: 1100 (perchlorate); 1156 (SO₂^{sym}), 1369 (SO₂^{as}), 1590 (C=C, C=N); 3060 (NH); ¹H-NMR (DMSO-*d*₆), δ, ppm: 2.46 (s, 6H, 2,6-Me₂); 7.01-7.49 (m, 9H, ArH, 1,4-phenylene bound to pyridinium + 4-Ph); 7.50-7.91 (m, 7H, ArH from phenoxathiin); 7.96 (s, 2H, ArH, 3,5-H from pyridinium);8.15 (s, 1H, SO₂NH); Anal. (C₃₆H₂₆N₂O₃S₂⁺ClO₄⁻) C, H, N.

5.5.39. *I-N-(Phenoxathiin-2-yl-aminosulfamoyl-phen-3-yl)*-2,4,6-triphenylpyridinium perchlorate **41**: yellow crystals, m.p. 235–236 °C. IR (KBr), cm⁻¹: 1100 (perchlorate); 1158 (SO₂^{sym}), 1379 (SO₂^{as}), 1596 (C=C, C=N); 3060 (NH); ¹H-NMR (DMSOd₆), δ, ppm: 6.92–7.54 (m, 19H, ArH, 1,3-phenylene bound to pyridinium + 3 Ph); 7.56–7.95 (m, 7H, ArH from phenoxathiin); 8.04 (s, 1H, SO₂NH); 8.55 (s, 2H, ArH, 3,5-H from pyridinium); Anal. (C₄₁H₂₈N₂O₃S₂+ClO₄⁻⁻) C, H, N.

5.5.40. 1-N-(Phenoxathiin-2-yl-aminosulfamoyl-phen-4-yl)-2,4,6-triphenylpyridinium perchlorate **42**: yellow crystals, m.p. 231–233 °C (dec). IR (KBr), cm⁻¹: 1100 (perchlorate); 1159 (SO₂^{sym}), 1366 (SO₂^{as}), 1595 (C=C, C=N); 3065 (NH); ¹H-NMR (DMSO- d_6), δ , ppm: 7.01–7.58 (m, 19H, ArH, 1,4-phenylene bound to pyridinium + 3 Ph); 7.54–7.96 (m, 7H, ArH from phenoxathiin); 8.05 (s, 1H, SO₂NH); 8.60 (s, 2H, ArH, 3,5-H from pyridinium); Anal. (C₄₁H₂₈N₂O₃S₂+ClO₄⁻) C, H, N.

5.6. Pharmacology

5.6.1. Assay of fungistatic activity of compounds 1-42

Fungistatic activity was determined by a modification of the growth method recently reported by us [27, 28], utilizing two *Aspergillus* and one *Candida spp*. Minimum inhibitory concentrations (MICs) have been determined by the agar dilution method with Iso-Sensitest agar as described by Kinsman et al. [29]. The fungi 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 100–0.01 µg/mL of compounds 1–42. The minimum concentration at which no growth was observed was taken as MIC value (µg/mL), and represents the mean of at least two determinations.

5.6.2. Assay of sterols present in the fungi cultures

A reverse-phase HPLC method adapted from the literature [30, 31] has been used to determine the amount of sterols (ergosterol 44 and lanosterol 43) present in the fungi cultures. The fungi have been 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 has been 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 of 3 mL/min. The retention times were: 8.87 min for ergosterol; and 7.62 min for lanosterol, respectively. Blank assay 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%. The alcohol, aldehyde and carboxy intermediates in the transformation of lanosterol to ergosterol have not been analyzed [33-36].

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