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Microwave-assisted synthesis and antimicrobial activity of 5-trihalomethyl-3-arylisoxazoles

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Abstract A series of twelve 5-trihalomethyl-3-arylisoxazoles was synthesized and screened for antibacterial and antifungal activities. The compounds were synthesized from the cyclondensation of 1,1,1-trihalo-4-alkoxy-3-alken-2-ones [$CX_3C(O)C(R^2)=C(R^1)OR$, where $X = Cl$ and F; $R = Me$; $R^2 = H$; $R^1 = H$, *Me*, F, Cl, Br, and NO_2] with hydroxylamine hydrochloride through a rapid one-pot reaction *via* microwave irradiation. Some of the 5-trihalomethyl-3-arylisoxazoles exhibited good *in vitro* anti-*Cryptococcus* activity.

Keywords Isoxazoles; Microwave irradiation; Antibacterial; Antifungal.

Introduction

There is no doubt that the existing arsenal of antimicrobial agents we have in hand for the treatment of infectious diseases will be insufficient over the long term [1]. The primary reason for this state of affairs is the inexorable drive of evolution that leads to antimicrobial resistance culminating with the failure of current treatments [1].

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In this context, fungal infections deserve special attention because fungi are emerging as important nosocomial pathogens causing severe morbidity and mortality in immunocompromised patients. Modern therapies and management such as bone marrow or solid-organ transplants, and new more aggressive chemotherapy have resulted in a rapidly expanding number of immunosuppressed patients. These patients now survive longer and become highly susceptible to life-threatening fungal infections [2]. Concomitant with the increased incidence of fungal infections has been a dramatic increase in the use of antifungals for the treatment of both systemic and localized fungal infections. Therefore, the expanded use of antifungal agents has accelerated the development of antifungal drug resistance, leading to frequent therapeutic failures and an increasing mortality rate [3].

For this reason, a constant effort toward the synthesis of new antifungal agents has been made over the last few years. The aim is to obtain novel leads which act through mechanisms of action distinct from those of well-known classes of antifungal agents, allowing for the treatment of many clinically relevant pathogens which are now resistant.

Compounds containing an isoxazole scaffold are known to possess antiinflammatory [4], antiviral [5],

antileukemic [6], and antagonist ionotropic glutamate receptor [7] activities, and are also modulators of the Multidrug Resistance Protein (MRP1) [8]. Literature has also reported some isoxazole derivatives endowed with antimicrobial activities [9–12], including the class of arylisoxazole derivatives [13, 14], however these studies have been limited in terms of microorganism strains.

Thus, the aim of this work is to show the synthesis of a series of 5-trihalomethyl-3-arylisoxazoles using environmentally benign microwave induced techniques. Furthermore, this work shows the screening of the synthesized compounds to observe their *in vitro* antibacterial and antifungal activities.

Results and discussion

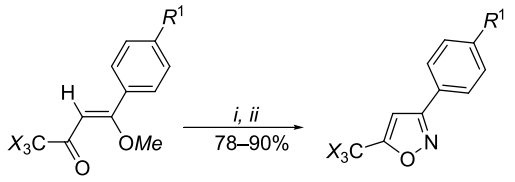
Chemistry

Over the last decade, we have developed a general synthesis of a large number of 1,1,1-trihalomethyl-4-methoxy-4-aryl[alkyl]-3-buten-2-ones, important halogen-containing building blocks, and demonstrated their usefulness in heterocyclic preparations [15]. As part of our research program there is an interest in improving the methodologies for the construction of heterocycles, and, therefore, we are investigating alternative methods for the preparation of these compounds, such as microwave irradiation [16] and ultrasound applications [17].

The beneficial effects of microwave irradiation are playing an increasing role in process chemistry, especially in cases where classical methods require forcing conditions or prolonged reaction times. Microwaves have also shown an advantage in processes involving sensitive reagents, or when there is the possibility of compound decomposition under prolonged reaction conditions. Reactions that require hours or even days by conventional heating can often be accomplished in seconds or minutes by microwave heating [18].

The 1,1,1-trihalomethyl-4-methoxy-4-aryl-3-buten-2-ones **1** and **2** were synthesized from the reaction of the respective acetal with trichloroacetyl chloride or trifluoroacetic anhydride [19, 20].

The 5-trihalomethyl-3-arylisoxazoles **3** and **4** were synthesized in two steps, using a one-pot process, as shown in Scheme 1. Firstly, enones **1** and **2** reacted with hydroxylamine hydrochloride in the presence of pyridine and using methanol as solvent. The solution was submitted to microwave irradiation (100 W) for 6 min, at a temperature of 70°C and at 2.2 bar of pressure, to produce the 5-trihalomethyl-5-hydroxy-4,5-dihydro-3-arylisoxazole intermediates. Secondly, after cooling to room temperature, conc. sulfuric acid was added and the mixture was again heated under



1a–1f, 2a–2f **3a–3f, 4a–4f**

Compound	1,3	2,4
X	Cl	F

Compound 1,2,3,4	a	b	c	d	e	f
R^1	H	*Me*	F	Cl	*Br*	NO_2

(i) $NH_2OH \cdot HCl$, *Me*OH, *Py*, MW, 100 W, 70°C, 2.2 bar, and 6 min; and (ii) H_2SO_4 conc., MW, 100 W, 80°C, 3.5 bar, and 10 min

Scheme 1

Table 1 Yields[a] and reaction time used for the microwave-assisted and conventional method for synthesis of **3a**–**3f** and **4a**–**4f**

Product	Microwave method		Conventional method[b]		Product	Microwave method		Conventional method[c]	
	Reaction time/min	Yield/%	Reaction time/h	Yield/%		Reaction time/min	Yield/%	Reaction time/h	Yield/%
3a	16	85	21	84	**4a**	16	87	48	67
3b	16	78	21	77	**4b**	16	83	48	70
3c	16	88	21	85	**4c**	16	85	48	69
3d	16	80	21	81	**4d**	16	80	48	73
3e	16	90	21	85	**4e**	16	80	48	68
3f	16	89	21	83	**4f**	16	85	48	80

[a] Yields of isolated products
[b] Reaction conditions: 1) $NH_2OH \cdot HCl$/pyridine/methanol/70°C/16 h; 2) H_2SO_4 conc./35°C/5 h
[c] Reaction conditions: $NH_2OH \cdot HCl$/HCl exc./methanol/70°C/48 h

microwave irradiation (100 W) for 10 min, at a temperature of 80°C to furnish the dehydrated 5-trihalomethyl-3-arylisoxazoles **3** and **4** with yields of 78–90%. The dehydration reaction of 4,5-dihydroazoles in acidic media is a second order elimination reaction ($E2_{(E1\text{-like})}$), where the stability of its activated complex depends on the participation of the electron pair of the neighboring oxygen atom (O-1) present in the isoxazole ring and of the electron donating effect of the group attached to the C-5 of the isoxazole ring.

This new method of forming of isoxazoles **3** and **4** under microwave irradiation offers several advantages: faster reaction rates, fewer byproducts, and good yields, while the conventional method involves moderate to good yields and a long tedious process, Table 1 [20]. For **3a–3f**, in the conventional method the cyclocondensation was carried out under reflux in methanol for 16 h followed by dehydration with sulfuric acid for 5 h [20]. Compounds **4a–4f** were obtained in a one-pot procedure using an excess of concentrate hydrochloric acid and stirring for 48 h [20]. The ^{1}H and ^{13}C NMR and physical data of all reported compounds are in full accordance with the data presented in Ref. [20].

Table 2 The *in vitro* antimicrobial profile of 5-trihalomethyl-3-arylisoxazoles **3a–3f** and **4a–4f** ($\mu g/cm^3$)

Comps.	*S. aureus*		*E. coli*		*P. aeruginosa*		*C. albicans*		*C. tropicalis*	
	MIC[a]	*MBC*[b]	*MIC*[a]	*MBC*[b]	*MIC*[a]	*MBC*[b]	*MIC*[a]	*MFC*[c]	*MIC*[a]	*MFC*[c]
3a	>625	–	>625	–	>625	>625	>625	–	312	–
3b	312	>625	>625	>625	156	>625	>625	–	>625	–
3c	>625	>625	>625	–	>625	>625	>625	–	>625	>625
3d	156	–	>625	–	>625	–	>625	–	312	–
3e	>625	>625	>625	>625	312	>625	156	–	>625	–
3f	312	>625	>625	>625	312	>625	>625	–	>625	–
4a	>625	–	>625	–	>625	–	312	–	>625	–
4b	>625	–	>625	–	>625	>625	312	>625	>625	–
4c	>625	–	–	–	>625	>625	156	–	>625	–
4d	>625	–	>625	>625	>625	>625	156	>625	312	>625
4e	312	>625	>625	–	>625	–	312	>625	>625	–
4f	>625	>625	>625	>625	>625	>625	156	–	>625	–
I	0.06		0.06		2.0					
F							4.0		4.0	

Comps.	*C. lusitaniae*		*C. neoformans* var. "*grubii*" A		*C. neoformans* var. "*gattii*" B		*C. neoformans* var. "*gattii*" C		*C. neoformans* D	
	MIC[a]	*MFC*[c]	*MIC*[a]	*MFC*[c]	*MIC*[a]	*MIC*[a]	*MFC*[c]	*MIC*[a]	*MFC*[c]	*MIC*[a]
3a	>625	–	156	>625	156	>625	–	156	>625	156
3b	156	–	39	>625	39	156	–	39	>625	39
3c	312	>625	156	>625	78	312	>625	156	>625	78
3d	312	–	156	–	156	312	–	156	–	156
3e	>625	–	156	>625	156	>625	–	156	>625	156
3f	>625	–	39	–	39	>625	–	39	–	39
4a	>625	–	156	>625	156	>625	–	156	>625	156
4b	>625	>625	39	>625	39	>625	>625	39	>625	39
4c	>625	>625	39	–	39	>625	>625	39	–	39
4d	39	>625	39	>625	39	39	>625	39	>625	39
4e	>625	–	39	>625	39	>625	–	39	>625	39
4f	312	>625	156	>625	156	312	>625	156	>625	156
I										
F	4.0		2.0		2.0	4.0		2.0		2.0

I Imipenem; *F* fluconazole; – no activity; *nt* not tested

[a] *MIC* Minimum inhibitory concentration ($\mu g/cm^3$)

[b] *MBC* Minimum bactericidal concentration ($\mu g/cm^3$)

[c] *MFC* Minimum fungicidal concentration ($\mu g/cm^3$)

Biological studies

The 5-trihalomethyl-3-arylisoxazoles were evaluated *in vitro* for antibacterial activity against representative human pathogenic *Gram*-positive bacterium, such as *Staphylococcus aureus* ATCC 25923 and *Gram*-negative bacterium, such as *Escherichia coli* ATCC 25322 and *Pseudomonas aeruginosa* ATCC 27853. The antifungal profile of the compounds was evaluated against a panel of fungi including *Candida albicans* ATCC 44373, *Candida tropicalis* ATCC 750, *Candida lusitaniae* ATCC 66035, *Cryptococcus neoformans* var. *grubii serotype* A ATCC 90012, *Cryptococcus neoformans* var. *gattii serotype* B ATCC 56990, *Cryptococcus neoformans* var. *gattii serotype* C ATCC 56341, and *Cryptococcus neoformans* var. *neoformans serotype* D ATCC 28957. Minimal Inhibitory Concentrations (*MIC*) were determined by means of a standard twofold dilution method and are reported in Table 2.

In this study, both *Gram*-positive cocci and *Gram*-negative rod susceptibilities to 5-trihalomethyl-3-arylisoxazoles only showed poor results. For Candida species, only **4d** exhibited good activity against *Candida lusitaniae*, being active at concentrations of $39\,\mu g/cm^3$.

The data depicted in Table 2 reveal that in contrast to the antibacterial behavior shown by these compounds, their antifungal effect was significant and pronounced. Particularly, *Cryptococcus* spp. seems to be more sensitive toward the synthesized 5-trihalomethyl-3-arylisoxazoles, as most of the compounds reported exhibited good activity against these microorganisms.

Cryptococcus neoformans is a yeast-like encapsulated fungus and an etiologic agent of meningoencephalitis affecting from 5 to 30% of AIDS patients, of which 10–25% die [21]. Therefore, the attainment of novel compounds able to inhibit cryptococcosis has been clinically relevant.

In general, anti-*Cryptococcus* activity was more pronounced for the 5-trifluoromethyl-3-arylisoxazoles (**4a**–**4f**). This differs from previous results obtained in our laboratories, where the trichloromethylated compounds, in general, showed higher activity than the trifluoromethylated analogues in carbamate based antimicrobial agents [22]. Another finding shows that the changes in the R^1 group induced only moderate differences in anti-*Cryptococcus* activity. For example, most of the compounds tested exhibited a *MIC* of $39\,\mu g/cm^3$ against *Cryptococcus neoformans* var. *gattii serotype* C. On the other hand, **3b** and **3f** showed *MIC*s of >625 and $39\,\mu g/cm^3$, against *Cryptococcus neoformans* var. *neoformans serotype* D. Thus, the replacement of methyl by a nitro group at R^1 of the 5-trichloromethyl-3-arylisoxazoles seems to have improved activity against this species of fungus. The comparison between *MIC*s and *MFC*s (minimal fungicidal concentrations) indicated that of 5-trihalomethyl-3-arylisoxazoles shown only fungistatic activity against *Cryptococcus* spp. as *MFC*s was obtained to be, in general, higher than *MIC*s by two or more concentrations.

In conclusion, the synthesized **3a**–**3f** and **4a**–**4f** were obtained with the use of microwave-assisted synthesis in a significantly shorter time (16 min) and with good yields (78–90%). Some of 5-trichloromethyl-3-arylisoxazoles showed good anti-*Cryptococcus* activity, thereby deserving additional studies due to their specificity for this species of fungi. These studies are in progress and include the determination of their activity against *Cryptococcus neoformans* isolates that show resistance to amphotericin B and 5-flucytosine or azole agents. Finally, the data obtained in this study suggest that the synthesized compounds could become promising prototypes for the future development of novel anti-*Cryptococcus* agents.

Experimental

Chemistry

Unless indicated otherwise, all common reagents were used as obtained from commercial suppliers without further purification. The solvents were dried and purified according to recommended procedures [23]. All melting points were measured using a *Reichert-Thermovar* apparatus. Yields listed are of isolated compounds. ^1H and ^{13}C NMR spectra were acquired on a *Bruker* DPX 200 or *Bruker* DPX 400 spectrometer (^1H at 200.13 or 400.13 MHz and ^{13}C at 50.32 or 100.63 MHz) at 300 K, 5 mm sample tubes, and with a digital resolution of ± 0.01 ppm. $CDCl_3$ was used as solvent with *TMS* as internal standard. Mass spectra were registered in a HP 5973 MSD connected to a HP 6890 GC and interfaced by a Pentium PC. The GC was equipped with a split-splitless injector, autosampler cross-linked HP-5 capillary column (30 m, 0.32 mm of internal diameter), and helium was used as the carrier gas. The reactions were performed in a multimode microwave ETHOS 1 (Milestone Inc.) which possesses a twin magnetron with a maximum delivered power of 1300 W. The temperature and the pressure were measured throughout with an ATC-400 CE and APC-55 detector.

General procedure for synthesis of 5-trihalomethyl-3-aryl-isoxazoles (**3a–3f**, **4a–4f**)

(1) A solution of **1** or **2** (2 mmol) and 167.7 mg hydroxylamine hydrochloride (2.4 mmol) in 4 cm^3 methanol and 0.5 cm^3 pyridine was stirred for a few minutes. The mixture was then irradiated in a microwave ETHOS 1 at 100 W, 2.2 bar of pressure for 6 min, enough time to complete the reaction. The temperature was set to 70°C and the irradiation was automatically stopped at this temperature.

(2) After cooling to room temperature, 4 cm^3 conc. sulfuric acid was added to the crude mixture which was again irradiated at 100 W, 3.5 bar of pressure for 10 min. The temperature was set to 80°C and the irradiation was automatically stopped at this temperature. The solution was then placed in 25 cm^3 cold water and the resulting solution was extracted with dichloromethane ($3 \times 20\,cm^3$). The organic phase was dried with $MgSO_4$, the solution was filtered, and the solvent was removed in a rotatory evaporator. The 5-trihalomethyl-3-arylisoxazoles **3a–3f** and **4a–4f** were obtained in high purity and, when necessary, the products were recrystallized from *n*-hexane. All products were found to be identical (m.p.s and spectroscopic properties) with those described earlier [20].

Biological assays

Minimal inhibitory concentrations (*MIC*) were determined by means of a standard twofold dilution method using broth medium; the antibacterial activity was based on the NCCLS M7-A5 document [24] and the evaluation of the antifungal activity was based on *Shadomy* and *Pfaller* [25] and the NCCLS M27-A2 document [26]. Test compounds were dissolved in *DMSO* at an initial concentration of 5000 $\mu g/cm^3$ and were then serially diluted in culture medium (*Müeller-Hinton* broth for bacterial and *Sabouraud* broth for fungal assays).

Cultures of microorganisms were adjusted to 10^5 *CFUs*/cm^3 according to the *McFarland* scale. Antimicrobial assays were carried out in triplicate and incubated at 35°C during 24 h for bacteria and *Candida* spp. and during 72 h for *Cryptococcus* spp. *MIC* was defined as the compound concentration at which no macroscopic sign of microbial growth was detected. The interpretation of the results was based on fluconazole breakpoints for the fungi and based on imipenem for bacterial pathogens, according to M27-A2 [25] and M7-A5 [23] techniques. The minimal germicidal concentrations (*MBC* or *MFC*) were determined by subcultivating samples from cultures with apparent growth in *Müeller-Hinton* agar for bacteria or *Sabouraud* dextrose agar for fungi.

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References

1. a) Brown ED, Wright GD (2005) Chem Rev 105:759; b) Coates A, Hu Y, Bax R, Page C (2002) Nat Rev Drug Discov 1:895
2. Espinel-Ingroff A, White T, Pfaller MA (1999) Antifungal Agents and Susceptibility Tests. In: Murray PR, Baron EJ, Pfaller MA, Tenover FC (eds) Manual of Clinical Microbiology, 7th edn. ASM Press, Washington, p 1640
3. Jorgensen JH, Turnidge JD, Washington JA (1999) Antibacterial Susceptibility Tests: Dilution and Disk diffusion methods. In: Murray PR, Baron EJ, Pfaller MA, Tenover FC, Yolken RH (eds) Manual of Clinical Microbiology, 7th edn. ASM Press, Washington, p 1526
4. Talley JJ, Brown DL, Carter JS, Graneto MJ, Koboldt CM, Masferrer JL, Perkins WE, Rogers RS, Shaffer AF, Zhang YY, Zweifel BS, Seibert KJ (2000) J Med Chem 43:775
5. Lee Y, Kim BH (2002) Bioorg Med Chem Lett 12:1395
6. Raffa D, Daidone G, Maggio B, Cascioferro S, Plescia F, Schillaci D (2004) Farmaco 59:451
7. Frølund B, Greenwood JR, Holm MM, Egebjerg J, Madsen U, Nielsen B, Bräuner-Osborne H, Stensbølc TB, Krogsgaard-Larsen P (2005) Bioorg Med Chem 13:5391
8. Norman BH, Lander PA, Gruber JM, Kroin JS, Cohen JD, Jungheim LN, Starling JJ, Law KL, Self TD, Tabas LB, Williams DC, Paul DC, Dantizig AH (2005) Bioorg Med Chem Lett 15:5526
9. Zadrozna I, Kurkowska J, Kruszewska H, Mukuch I (2000) Farmaco 55:499
10. Raffa D, Daidone G, Maggio B, Scillaci D, Plescia F, Torta L (1999) Farmaco 54:90
11. Calí P, Naerum L, Mukhija S, Hjelmencrantz A (2004) Bioorg Med Chem Lett 14:5997
12. Aiello E, Aiello S, Mingoia F, Bacchi A, Pelizzi G, Musiu C, Setzu M G, Pani A, La Colla P, Marongiu E (2000) Bioorg Med Chem 8:2719
13. Davenport JD, Dreikorn BA, Elsasser AF (1976) German Patent 2723688; (1978) Chem Abstr 88:132015k
14. Barbachyn MR, Cleek GJ, Dolak LA, Garmon SA, Morris J, Seest EP, Thomas RC, Toops DS, Watt W, Wishka DG, Ford CW, Zurenko GE, Hamel JC, Schaadt RD, Stapert D, Yagi BH, Adams WJ, Friis JM, Slatter JG, Sams JP, Oien NL, Zaya MJ, Wienkers LC, Wynalda MA (2003) J Med Chem 46:284
15. Martins MAP, Cunico W, Pereira CMP, Flores AFC, Bonacorso HG, Zanatta N (2004) Curr Org Synthesis 1:391; and references therein
16. a) Martins MAP, Pereira CMP, Beck P, Machado P, Brondani S, Moura S, Teixeira MVM, Bonacorso HG, Zanatta N (2003) Tetrahedron Lett 44:6669; b) Martins MAP, Beck P, Machado P, Brondani S, Moura S, Zanatta N, Bonacorso HG, Flores AFC (2006) J Braz Chem

Soc 17:408; c) Sauzem PD, Machado P, Rubin MA, Sant'Anna GS, Faber HB, Souza AH, Mello CF, Beck P, Burrow RA, Bonacorso HG, Zanatta N, Martins MAP (2007) Eur J Med Chem (in press)
17. Martins MAP, Pereira CMP, Cunico W, Moura S, Rosa FA, Peres RL, Machado P, Zanatta N, Bonacorso HG (2006) Ultrasonics Sonochem 13:364
18. Larhed M, Moberg C, Hallberg A (2002) Acc Chem Res 35:717; and references therein
19. a) Colla A, Martins MAP, Clar G, Krimmer S, Fischer P (1991) Synthesis 6:483; b) Martins MAP, Bastos GP, Bonacorso HG, Zanatta N, Flores AFC, Siqueira GM (1999) Tetrahedron Lett 40:4309; c) Bonacorso HG, Martins MAP, Bittencourt SRT, Lourega RV, Zanatta N, Flores AFC (1999) J Fluorine Chem 99:177
20. Martins MAP, Siqueira GM, Bastos GP, Bonacorso HG, Zanatta N (1996) J Heterocycl Chem 33:1619
21. Viviani MA, Tortorano AM, Ajello L (2003) Cryptococcus. In: Anaissie EJ, McGinnis MR, Pfaller MA (eds) Clinical Mycology. Churchil Livingstone, New York, p 240
22. Zanatta N, Borchhardt DM, Alves SH, Coellho HS, Squizani AMC, Marchi TM, Bonacorso HG, Martins MAP (2006) Bioorg Med Chem 14:3174
23. Perrin DD, Armarego WLF (1998) Purification of Laboratory Chemicals. Pergamon, Oxford
24. National Committee for Clinical Laboratory Standards (2002) Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically, Approved Standard, 5th edn. NCCLS document M7-A5, Wayne, P. A. National Committee for Clinical Laboratory Standards
25. Shadomy S, Pfaller M (1991) Laboratory studies with antifungal agents: susceptibility tests and quantitation in sody fluids. In: Ballours A, Hauster WJ Jr, Herrmanx KL, Isemberg HD, Shadomy HJ (eds) Manual of Clinical Microbiology, 5th edn. Americam Society of Microbiology, Washington, p 1173
26. National Committee for Clinical Laboratory Standards (2002) Reference method for broth dilution antifungal susceptibility testing of yeast: approved standard. NCCLS document M27-A2. Wayne, P. A. National Committee for Clinical Laboratory Standards