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Antifungal Activity of Xanthones: Evaluation of their Effect on Ergosterol Biosynthesis by High-performance Liquid Chromatography

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The increasing resistance of pathogenic fungi to antifungal compounds and the reduced number of available drugs led to the search for therapeutic alternatives among natural products, including xanthones. The antifungal activity of 27 simple oxygenated xanthones was evaluated by determination of their minimal inhibitory concentration on clinical and type strains of *Candida*, *Cryptococcus*, *Aspergillus* and dermatophytes, and their preponderance on the dermatophytic filamentous fungi was observed. Furthermore, a simple and efficient HPLC method with UV detection to study the effect of the active xanthones on the biosynthesis of ergosterol was developed and validated. Using this methodology, the identification and quantification of fungal sterols in whole cells of *Candida albicans*, *Cryptococcus neoformans*, *Aspergillus fumigatus*, and *Trichophyton mentagrophytes* were accomplished. In summary, 1,2-dihydroxyxanthone was found to be the most active compound against all strains tested, showing its effect on sterol biosynthesis by reducing the amount of ergosterol detected.

Key words: antifungal, *Aspergillus*, *Candida*, *Cryptococcus*, dermatophytes, ergosterol, xanthone

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It is well recognized that nowadays invasive mycoses have become important causes of morbidity and mortality in immunocompromised patients, such as those infected with HIV and those receiving cancer chemotherapy, immunosuppressive therapy, or treatment with broad-spectrum antibiotics (1). The most frequently encountered infections are caused by the yeast *Candida albicans* and also by species of the filamentous fungus *Aspergillus* (2). On the other hand, dermatophytes (the genera *Epidermophyton*, *Trichophyton*, and *Microsporum*), which invade and multiply in keratinized tissues (3) and cause infection, have a worldwide distribution with geographic differences in the incidence and prevalence (4).

The majority of clinically used antifungal drugs suffer from various drawbacks in terms of toxicity, efficacy, and cost, as well as the emergence of resistant strains caused by their frequent use (5). Although this was counteracted by new advances in molecular genetics which have afforded the promise of revealing new antifungal targets together with new agents to inhibit those targets specifically (6), there is still a great demand for novel and effective antifungal agents. One of the most promising groups of antifungal agents is the xanthones (9*H*-xanthen-9-ones), which are heterocyclic compounds based on the dibenzo-γ-pyrone scaffold (Figure 1). Nowadays, xanthone derivatives are of relevance because of the several biologic activities reported (7). Concerning antifungal activity, investigations have been undertaken in the group of both synthetic (8,9) and naturally occurring xanthone derivatives. Consequently, in an attempt to search for naturally occurring antifungal compounds, a number of xanthones with clinical value against fungal infections, from higher plants (8,10–26) and microorganisms (27–34) including marine species (35,36), have been identified. These xanthones are mainly divided into four subclasses: simple oxygenated (8,15,17,25,26,35,36), prenylated (8,10–14,16–22,24,26), polycyclic (27–29), and dehydroxanthones (15,31–34) such as ergochromes and hemisecalonic acids. Some of these compounds have revealed significant antifungal properties, e.g., Sch 54445 that exhibits highly potent activities against various yeasts and dermatophytes (27). Nonetheless, little information is available on structure–antifungal

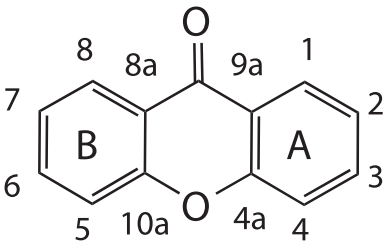

Figure 1: Xanthone core and numbering.

activity relationship of oxygenated xanthones and ultimately on their mechanism of action.

Several bioactive xanthones, different in nature and pattern of substitution in one of the aromatic rings, have already been reported by our group for their antitumor (37–40), immunomodulatory (38,41–43), modulatory activity of protein kinase C (44–46), antimalarial (47), hepatoprotective (48), and monoaminoxidase inhibitory effects (49,50). Following up our investigation into bioactive xanthones, we have evaluated 27 hydroxylated/methoxylated xanthone derivatives for their antifungal activity. Thus, the aims of this work are to evaluate the influence of the nature and position of the substituents on structure–activity relationships of antifungal xanthones and to investigate the effect of the most active compounds on sterol biosynthesis. The insight into their mechanisms of action can not only provide important information in an attempt to improve the antifungal activity of these compounds, but also allow their combination with other antifungal agents in therapeutics.

On the one hand, it is well established that ergosterol is the predominant sterol in fungal cell membrane and is responsible for maintaining cell integrity and function as well as for the normal growth. Thus, most of the current frontline antifungal agents act either on ergosterol biosynthesis (azoles, allylamines) or on ergosterol itself (amphotericin B). GC-MS is the most current method used for the quantification of sterols. Although this method provides greater sensitivity, it requires sample derivatization. Consequently, it is very complex and takes longer time to execute. We hereby report the development of a simpler and quicker method to separate and calculate the sterol content in fungal whole cell assays using a normal-phase HPLC without prior tetramethylsilane (TMS) derivatization (51).

Experimental Section

Chemistry

Melting points were obtained in a Köfler microscope and are uncorrected. IR spectra were recorded on a Perkin Elmer 257 in KBr. ^{1}H and ^{13}C NMR spectra were taken in DMSO-d_6 at room temperature, on Bruker DRX 300 instrument. Chemical shifts are expressed in δ (ppm) values relative to TMS. HR-MS results were obtained in CACTI services, Vigo, Spain.

Xanthone (**1**) and 2,2′,4,4′-tetrahydroxybenzophenone (**28**) were purchased from Sigma Chemical Co., St. Louis, USA. The natural products 2-hydroxy-1-methoxyxanthone (**24**), 1,7-dihydroxyxanthone (**25**), 2-hydroxy-1,8-dimethoxyxanthone (**26**), and 1,2,8-trimethoxyxanthone (**27**) were kindly provided by Prof. Anake Kijjoa, ICBAS-Instituto de Ciências Biomédicas de Abel Salazar, Portugal, and were isolated from *Calophyllum teysmannii* var. *inophylloide* (41,52). The following xanthone derivatives were synthesized according to previously described procedures (38,49,53).

1-Hydroxyxanthone (2) 48%yield. ^{1}H-NMR ((D$_6$)DMSO) δ: 12.56 (s, OH-C(1)), 8.18 (dd, J = 8.2, 1.7, H-C(8)), 7.92 (ddd, J = 8.1, 7.6, 1.7, H-C(6)), 7.74 (dd, J = 8.3, 8.2, H-C(3)), 7.67 (d, J = 8.1, H-C(5)), 7.51 (dd, J = 8.2, 7.6, H-C(7)), 6.83 (dd, J = 8.3, 0.6, H-C(2)). ^{13}C-NMR ((D$_6$)DMSO): 181.8 (C(9)), 161.0 (C(1)), 155.8 (C(4a)), 155.7 (C(10a)), 137.6 (C(3)), 136.5 (C(6)), 125.5 (C(8)), 124.7 (C(7)), 119.9 (C(8a)), 118.1 (C(5)), 110.2 (C(2)), 108.4 (C(9a)), 107.3 (C(4)).

2-Hydroxyxanthone (3) 74%yield. ^{1}H-NMR ((D$_6$)DMSO) δ: 10.00 (s, OH-C(2)), 8.19 (dd, J = 8.0, 1.7, H-C(8)), 7.85 (ddd, J = 8.2, 7.6, 1.7, H-C(6)), 7.63 (dd, J = 8.2, 0.9, H-C(5)), 7.56 (d, J = 9.0, H-C(4)), 7.48 (d, J = 3.0, H-C(1)), 7.45 (ddd, J = 8.0, 7.6, 0.9, H-C(7)), 7.32 (dd, J = 9.0, 3.0, H-C(3)). ^{13}C-NMR ((D$_6$)DMSO): 175.9 (C(9)), 155.6 (C(10a)), 153.9 (C(2)), 149.2 (C(4a)), 135.2 (C(6)), 125.9 (C(8)), 124.6 (C(3)), 124.0 (C(7)), 121.7 (C(9a)), 120.4 (C(8a)), 119.5 (C(4)), 118.2 (C(5)), 108.5 (C(1)).

3-Hydroxyxanthone (4) 92%yield. ^{1}H-NMR ((D$_6$)DMSO) δ: 11.00 (s, OH-C(3)), 8.15 (dd, J = 7.9, 1.7, H-C(8)), 8.04 (d, J = 8.6, H-C(1)), 7.82 (ddd, J = 8.2, 7.6, 1.7, H-C(6)), 7.61 (d, J = 8.2, H-C(5)), 7.44 (dd, J = 7.9, 7.6, H-C(7)), 6.91 (dd, J = 8.6, 2.2, H-C(2)), 6.88 (d, J = 2.2, H-C(4)). ^{13}C-NMR ((D$_6$)DMSO): 174.8 (C(9)), 164.0 (C(3)), 157.6 (C(4a)), 155.6 (C(10a)), 134.9 (C(6)), 128.0 (C(1)), 125.9 (C(8)), 124.2 (C(7)), 121.2 (C(8a)), 117.9 (C(5)), 114.2 (C(2)), 114.0 (C(9a)), 102.1 (C(4)).

4-Hydroxyxanthone (5) 59%yield. ^{1}H-NMR ((D$_6$)DMSO) δ: 10.51 (s, OH-C(4)), 8.19 (dd, J = 7.8, 1.6, H-C(8)), 7.88 (ddd, J = 8.0, 7.6, 1.6, H-C(6)), 7.73 (dd, J = 8.0, 0.8, H-C(5)), 7.61 (dd, J = 7.8, 1.8, H-C(1)), 7.48 (ddd, J = 7.8, 7.6, 0.8, H-C(7)), 7.34 (dd, J = 7.8, 1.8, H-C(3)), 7.26 (t, J = 7.8, H-C(2)). ^{13}C-NMR ((D$_6$)DMSO): 176.2 (C(9)), 155.4 (C(10a)), 146.7 (C(4)), 145.2 (C(4a)), 135.4 (C(6)), 126.0 (C(8)), 124.3 (C(7)), 124.1 (C(2)), 122.2 (C(9a)), 120.9 (C(8a)), 120.2 (C(3)), 118.3 (C(5)), 115.2 (C(1)).

1-Methoxyxanthone (6) 86%yield. ^{1}H-NMR ((D$_6$)DMSO) δ: 8.09 (dd, J = 7.7, 1.6, H-C(8)), 7.80 (ddd, J = 7.8, 7.6, 1.6, H-C(6)), 7.74 (dd, J = 8.4, 8.3, H-C(3)), 7.57 (dd, J = 7.8, 0.7, H-C(5)), 7.42 (ddd, J = 7.7, 7.6, 0.7, H-C(7)), 7.00 (d, J = 8.3, H-C(2)), 3.91 (s, MeO-C(1)). ^{13}C-NMR ((D$_6$)DMSO): 174.7 (C(9)), 160.2 (MeO-C(1)), 157.4 (C(4a)), 154.4 (C(10a)), 135.7 (C(3)), 134.8 (C(6)), 125.9 (C(8)), 124.2 (C(7)), 122.4 (C(8a)), 117.5 (C(5)), 111.6 (C(9a)), 109.6 (C(4)), 106.4 (C(2)).

2-Methoxyxanthone (7) 66%yield. ^{1}H-NMR ((D$_6$)DMSO) δ: 8.18 (dd, J = 7.7, 1.7, H-C(8)), 7.86 (ddd, J = 8.2, 7.4, 1.7, H-C(6)), 7.64 (dd, J = 8.2, 1.9, H-C(5)), 7.63 (d, J = 9.1, H-C(4)), 7.54 (d, J = 3.2, H-C(1)), 7.47 (dd, J = 9.1, 3.2, H-C(3)), 7.46 (ddd, J = 7.7, 7.4, 1.0, H-C(7)), 3.87 (s, MeO-C(2)). ^{13}C-NMR ((D$_6$)DMSO): 175.8 (C(9)), 155.7 (C(2)), 155.5 (C(10a)), 150.3 (C(4a)), 135.4 (C(6)), 126.0 (C(8)), 124.7 (C(3)), 124.2 (C(7)), 121.5 (C(9a)), 120.5 (C(8a)), 119.8 (C(4)), 118.2 (C(5)), 105.7 (C(1)), 55.7 (MeO-C(2)).

3-Methoxyxanthone (8) 45%yield. ^{1}H-NMR ((D$_6$)DMSO) δ: 8.17 (dd, J = 7.7, 1.7, H-C(8)), 8.10 (d, J = 8.9, H-C(1)), 7.85 (ddd, J = 8.0, 7.6, 1.7, H-C(6)), 7.63 (d, J = 8.0, H-C(5)), 7.47 (dd, J = 7.7, 7.6, H-C(7)), 7.16 (d, J = 2.4, H-C(4)), 7.05 (dd, J = 8.9, 2.4, H-C(2)), 3.93 (s, MeO-C(3)). ^{13}C-NMR ((D$_6$)DMSO, 75.47 MHz): 174.9 (C(9)), 165.0 (C(3)), 157.6 (C(4a)), 155.6 (C(10a)), 135.1 (C(6)), 127.6 (C(1)), 125.9 (C(8)), 124.4 (C(7)), 121.2 (C(8a)), 117.9 (C(5)), 114.9 (C(9a)), 113.7 (C(2)), 100.6 (C(4)), 56.2 (MeO-C(3)).

4-Methoxyxanthone (9) 14%yield. ^{1}H-NMR ((D$_6$)DMSO) δ: 8.20 (dd, J = 8.0, 1.6, H-C(8)), 7.88 (dt, J = 8.1, 1.6, H-C(6)), 7.73 (dd, J = 7.9, 1.4, H-C(1)), 7.72 (d, J = 8.1, H-C(5)), 7.52 (dd, J = 7.9, 1.4, H-C(3)), 7.49 (dd, J = 8.0, 7.8, H-C(7)), 7.40 (t, J = 7.9, H-C(2)), 3.99 (s, MeO-C(4)). ^{13}C-NMR ((D$_6$)DMSO): 176.0 (C(9)), 155.4 (C(10a)), 148.4 (C(4)), 145.8 (C(4a)), 135.5 (C(6)), 125.9 (C(8)), 124.5 (C(7)), 124.0 (C(2)), 121.0 (C(8a)), 121.9 (C(9a)), 118.4 (C(5)), 116.4 (C(3)), 116.4 (C(1)), 56.2 (MeO-(C(4)).

1,2-Dihydroxyxanthone (10) 48%yield. ^{1}H-NMR ((D$_6$)DMSO) δ: 12.45 (OH-C(1)), 9.42 (OH-C(2)), 8.16 (dd, J = 7.9, 1.6, H-C(8)), 7.81 (ddd, J = 8.4, 7.0, 1.6, H-C(6)), 7.60 (dd, J = 8.4, 0.8, H-C(5)) 7.46 (ddd, J = 7.9, 7.0, 0.8 H-C(7)), 7.32 (d, J = 9.0, H-C(4)), 6.96 (d, J = 9.0, H-C(3)), ^{13}C-NMR ((D$_6$)DMSO): 182.3 (C(9)), 155.9 (C(10a)), 148.3 (C(4a)), 147.6 (C(1)), 140.2 (C(2)), 136.4 (C(6)), 125.4 (C(8)), 124.5 (C(3)), 124.2 (C(7)), 119.3 (C(8a)), 118.0 (C(5)), 108.8 (C(9a)), 106.2 (C(4)).

2,3-Dihydroxyxanthone (11) 90%yield. ^{1}H-NMR ((D$_6$)DMSO) δ: 8.12 (dd, J = 7.5, 1.6, H-C(8)), 7.78 (ddd, J = 8.4, 6.8, 1.7, H-C(6)), 7.58 (d, J = 8.4 Hz, H-C(5)), 7.43 (s, H-C(1)), 7.42 (ddd, J = 7.5, 6.8, 1.9 Hz, H-C(7)), 6.92 (s, H-C(4)), ^{13}C-NMR ((D$_6$)DMSO): 175.0 (C(9)), 155.7 (C(10a)), 154.2 (C(4a)), 151.3 (C(3)), 144.1 (C(2)), 134.7 (C(6)), 125.9 (C(8)), 124.1 (C(7)), 120.9 (C(8)), 118.1 (C(5)), 113.7 (C(9a)), 108.9 (C(1)), 103.0 (C(4)).

3,4-Dihydroxyxanthone (12) 78%yield. ^{1}H-NMR ((D$_6$)DMSO) δ: 8.14 (dd, J = 8.1, 1.6, H-C(8)), 7.81 (ddd, J = 8.6, 6.9, 1.7, H-C(6)), 7.62 (dd, J = 8.6, 0.9, H-C(5)), 7.56 (d, J = 8.6, H-C(1)), 7.42 (ddd, J = 8.1, 6.9, 0.9, H-C(7)), 6.93 (d, J = 8.6, H-C(2)), ^{13}C-NMR ((D$_6$)DMSO): 175.3 (C(9)), 155.5 (C(10a)), 151.6 (C(3)), 146.4 (C(4a)), 134.8 (C(6)), 132.7 (C(4)), 125.9 (C(8)), 124.0 (C(7)), 120.8 (C(8a)), 118.0 (C(5)), 116.6 (C(1)), 114.7 (C(9a)), 113.2 (C(2)).

1,2-Dimethoxyxanthone (13) 74%yield. ^{1}H-NMR ((D$_6$)DMSO) δ: 8.12 (dd, J = 7.9 and 1.7, H-C(8)), 7.81 (ddd, J = 7.6, 7.4, 1.7, H-C(6)), 7.63 (d, J = 9.3, H-C(4)), 7.56 (d, J = 7.4, H-C(5)), 7.44 (dd, J = 7.9, 7.6, 1.1, H-C(7)), 7.40 (d, J = 9.3, H-C(3)), 3.86 (s, MeO-C(1)), 3.80 (s, MeO-C(2)), ^{13}C-NMR ((D$_6$)DMSO): 175.2 (C(9)), 154.7 (C(10a)), 150.3 (C(2)), 148.9 (C(1)), 147.5 (C(4a)), 135.0 (C(6)), 126.0 (C(8)), 123.9 (C(7)), 121.4 (C(8a)), 120.7 (C(5)), 117.6 (C(3)), 116.4 (C(9a)), 113.2 (C(4)), 61.0 (MeO-C(1)), 56.6 (MeO-C(2)).

2,3-Dimethoxyxanthone (14) 61%yield. ^{1}H-NMR ((D$_6$)DMSO) δ: 8.16 (dd, J = 7.9, 1.5, H-C(8)), 7.82 (ddd, J = 8.4, 7.4, 1.5, H-C(6)), 7.60 (d, J = 8.4, H-C(5)), 7.49 (s, H-C(1)), 7.44 (ddd, J = 7.9, 7.4, 0.9 H-C(7)), 7.20 (s, H-C(4)), 3.94 (s, MeO-C(3)), 3.87 (3H, s, MeO-C(2)), ^{13}C-NMR ((D$_6$)DMSO): 174.8 (C(9)), 155.7 (C(10a)), 155.6 (C(4a)), 152.0 (C(3)), 146.7 (C(2)), 134.8 (C(6)), 125.9 (C(8)), 124.3 (C(7)), 120.9 (C(8)), 118.0 (C(5)), 114.0 (C(9a)), 104.7 (C(1)), 100.5 (C(4)), 56.6 (MeO-C(3)), 55.9 (MeO-C(2)).

3,4-Dimethoxyxanthone (15) 61%yield. ^{1}H-NMR ((D$_6$)DMSO) δ: 8.16 (dd, J = 7.9, 1.7, H-C(8)), 7.94 (d, J = 9.0, H-C(1)), 7.85 (ddd, J = 8.1, 7.5, 1.7, H-C(6)), 7.69 (d, J = 8.1, H-C(5)), 7.47 (dd, J = 7.9, 7.5, H-C(7)), 7.26 (d, J = 9.0, H-C(2)), 3.97 (s, MeO-C(3)), 3.92 (s, Me-C(4)). ^{13}C-NMR ((D$_6$)DMSO): 175.3 (C(9)), 157.5 (C(3)), 155.6 (C(10a)), 149.9 (C(4a)), 135.9 (C(4)), 135.2 (C(6)), 125.9 (C(8)), 124.4 (C(7)), 121.7 (C(1)), 120.8 (C(8a)), 118.2 (C(5)), 115.9 (C(9a)), 109.7 (C(2)), 60.9 (MeO-C(4)), 56.1 (MeO-C(3)).

3-Hydroxy-4-methoxyxanthone (16) 51%yield. ^{1}H-NMR ((D$_6$)DMSO) δ: 10.79 (s, OH-C(3)), 8.16 (dd, J = 7.7, 1.7, H-C(8)), 7.84 (ddd, J. 8.1, 7.6, 1.7, H-C(6)), 7.80 (d, J.8.9, H-C(1)), 7.69 (dd, J.8.1, 1.0, H-C(5)), 7.46 (ddd, J.7.7, 7.6, 1.0, H-C(7)), 7.00 (d, J.8.9, H-C(2)), 3.92 (s, MeO-C(4)). ^{13}C-NMR ((D$_6$)DMSO): 175.0 (C(9)), 156.3 (C(3)), 155.5 (C(10a)), 150.7 (C(4a)), 135.0 (C(6)), 134.6 (C(4)), 125.9 (C(8)), 124.3 (C(7)), 121.6 (C(1)), 120.9 (C(8a)), 118.2 (C(5)), 114.8 (C(9a)), 114.1 (C(2)), 60.9 (MeO-C(4)).

4-Hydroxy-3-methoxyxanthone (17) 39%yield. ^{1}H-NMR ((D$_6$)DMSO) δ: 9.66 (s, OH-C(4)), 8.16 (dd, J = 7.8, 1.7, H-C(8)), 7.84 (ddd, J = 8.3, 7.6, 1.7, H-C(6)), 7.68 (d, J = 9.0, H-C(1)), 7.65 (dd, J = 8.3, 1.0, H-C(5)), 7.44 (ddd, J = 7.8, 7.6, 1.0, H-C(7)), 7.19 (d, J = 9.0, H-C(2)), 3.96 (s, MeO-C(3)). ^{13}C-NMR ((D$_6$)DMSO): 175.6 (C(9)), 155.7 (C(10a)), 152.5 (C(3)), 145.5 (C(4a)), 135.1 (C(6)), 134.1 (C(4)), 126.0 (C(8)), 124.1 (C(7)), 120.8 (C(8a)), 118.1 (C(5)), 116.3 (C(1)), 115.9 (C(9a)), 109.0 (C(2)), 56.4 (MeO-C(3)).

3,5-Dihydroxyxanthone (18) 55%yield. ^{1}H-NMR ((D$_6$)DMSO) δ: 8.02 (d, J = 9.4, H-C(1)), 7.56 (dd, J = 7.5, 1.8, H-C(8)), 7.27 (dd, J = 7.8, 1.8, H-C(6)), 7.21 (t, J = 7.7, H-C(7)), 6.89 (dd, J = 7.4, 2.1, H-C(2)), 6.88 (s, H-C(4)). ^{13}C-NMR ((D$_6$)DMSO): 175.0 (C(9)), 163.9 (C(3)), 157.3 (C(4a)), 146.3 (C(5)), 145.8 (C(10a)), 127.9 (C(1)), 123.8 (C(7)), 122.0 (C(8a)), 116.1 (C(6)), 115.2 (C(8)), 114.2 (C(2)), 113.8 (C(9a)), 102.1 (C(4)).

3,5-Dimethoxyxanthone (19) 35%yield. ^{1}H-NMR ((D$_6$)DMSO) δ: 8.09 (d, J = 8.9, H-C(1)), 7.70 (dd, J = 7.9, 1.7, H-C(8)), 7.49 (dd, J = 7.9, 1.7, H-C(6)), 7.38 (t, J = 7.9, H-C(7)), 7.20 (d, J = 2.4, H-C(4)), 7.05 (dd, J = 8.8, 2.4, H-C(2)), 3.97 (s, MeO-C(5)), 3.94 (s, MeO-C(3)). ^{13}C-NMR ((D$_6$)DMSO): 175.0 (C(9)), 165.0 (C(3)), 157.4 (C(4a)), 148.3 (C(5)), 145.8 (C(10a)), 127.5 (C(1)), 124.0 (C(7)), 122.0 (C(8a)), 116.4 (C(8)), 116.1 (C(6)), 114.8 (C(2)), 114.1 (C(9a)), 100.6 (C(4)), 56.2 (MeO-C(3), MeO-C(5)).

3-Hydroxy-5-methoxyxanthone (20) 42%yield. ^{1}H-NMR ((D$_6$)DMSO) δ: 8.03 (d, J = 8.6, H-C(1)), 7.70 (dd, J = 7.9, 1.2, H-C(8)), 7.46 (dd, J = 7.9, 1.2, H-C(6)), 7.35 (t, J = 7.9, H-C(7)), 6.91 (dd, J = 8.6, 2.0, H-C(2)), 6.88 (d, J = 2.0, H-C(4)), 3.96 (s, MeO-C(5)). ^{13}C-NMR ((D$_6$)DMSO): 174.8 (C(9)), 164.1 (C(3)), 157.4 (C(4a)), 148.2 (C(5)), 145.7 (C(10a)), 128.0 (C(1)), 123.8 (C(7)), 122.0 (C(8a)), 116.4 (C(8)), 115.9 (C(6)), 114.4 (C(2)), 113.9 (C(9a)), 102.24 (C(4)), 55.2 (MeO-C(5)).

1,3-Dimethoxyxanthone (21) 30%yield. ^{1}H-NMR ((D$_6$)DMSO) δ: 8.06 (dd, J = 7.7, 1.7, H-C(8)), 7.75 (ddd, J = 7.9, 7.0, 1.8, H-C(6)), 7.51 (dd, J = 7.9, 1.8, C(5)), 7.39 (ddd, J = 7.9, 7.0, 1.8, H-C(7)), 6.69 (d, J = 2.2, C(4)), 6.50 (d, J = 2.2, H-C(2)), 3.90 (s, MeO-C(1)), 3.86 (s, MeO-C(3)). ^{13}C-NMR ((D$_6$)DMSO, 200 MHz): 180.0 (C.O), 164.8 (C(3)), 161.5 (C(1)), 159.1 (C(4a)), 154.3 (C(10a)), 134.3 (C(6)), 125.9 (C(8)), 124.1 (C(7)), 122.4 (C(8a)), 117.2 (C(5)), 107.9 (C(9a)), 95.4 (C(2)), 93.2 (C(4)), 56.2 (MeO-C(1)), 56.0 (MeO-C(3)).

2,7-Dimethoxyxanthone (22) 40%yield. ^{1}H-NMR ((D$_6$)DMSO) δ: 7.45 (dd, J = 9.0, 3.0, H-C(3) and H-C(6)), 7.33 (d, J = 9.1, H-4 and

H-5), 7.80 (d, *J* = 9.0, H-C(1) and H-C(8)), 3.87 (s, MeO-C(2), MeOH-C(7)). ^{13}C-NMR ((D$_6$)DMSO): 175.5 (C(9)), 155.6 (C(2), C(7)), 150.3 (C(4a) C(10a)), 124.7 (C(3), C(6)), 120.8 (C(8a), C(9a)), 119.8 (C(1), C(4)), 55.7 (MeO-C(2), MeOH-C(7)).

Synthesis of 3,6-dihydroxyxanthone (23)

The heating of 2,2′,4,4′-tetrahydroxybenzophenone (**28**, 500 mg; 2 mmol) at 180 °C, in a furnace, overnight, afforded the pure product.

23. Brown solid. 85% yield. Mp > 330/C; IR (KBr): 3383; 3135; 1610, 1579, 1454; 1251; 1170 cm^{-1}; ^{1}H-NMR ((D$_6$)DMSO) δ: 10.88 (s, OH-C(3) and OH-C(6)), 8.00 (d, J = 8.7, H-C(1) and H-C(8)), 6.88 (dd, J = 8.7, 2.2, H-C(2) and H-C(7)), 6.84 (d, J = 2.2, H-C(4) and H-C(5)). ^{13}C NMR ((D$_6$)DMSO): 174.0 (C(9)), 163.4 (C(3), C(6)), 157.5 (C(4a), C(10a)), 127.8 (C(1), C(8)), 114.0 (C(8a), C(9a)), 113.7 (C(2), C(7)), 102.1 (C(4), C(5)). HRMS-FAB$^+$ *m/z* calcd for $C_{13}H_8O_4^+$: 229.0501, found: 229.0504.

Microorganisms

The antifungal activity of the xanthones was evaluated against *Candida*, *Cryptococcus*, *Aspergillus*, and dermatophyte strains: clinical isolates (*C. glabrata* D10R, from recurrent cases of oral candidosis; *Cryptococcus neoformans* PH1, from cerebrospinal fluid; *Microsporum canis* FF1, *M. gypseum* FF3, *Trichophyton mentagrophytes* FF7, *T. rubrum* FF5, and *Epidermophyton floccosum* FF9, all isolated from nails and skin) and ATCC (American Type Culture Collection) type strains (*C. albicans* ATCC 10231 and *Aspergillus fumigatus* ATCC 46645). Strains were stored in Sabouraud dextrose broth with glycerol, at −70 °C. To ensure optimal growth, they were subcultured twice in Sabouraud dextrose agar (SDA) with chloramphenicol (BioMérieux) prior to testing and the cultures were incubated during 24 h (for *Candida* spp.), 48 h (for *C. neoformans* and *A. fumigatus*), and 7 days for dermatophytes.

Susceptibility testing

Broth microdilution methods based on the CLSI (formerly NCCLS) reference documents M27A-2 (54) and M38-A (55) for yeasts and filamentous fungi, respectively, with minor modifications, were used to determine minimum inhibitory concentrations (MIC).

Briefly, cell or spore suspensions were prepared from recent cultures on SDA with chloramphenicol of the different strains of fungi and diluted to final inoculum sizes of 10^3 CFU (colony forming units) mL^{-1} in RPMI-1640 broth (Sigma), with glutamine, without bicarbonate and with phenol red as the pH indicator, buffered to pH 7.0 with MOPS (Sigma). Serial twofold dilutions of each xanthone (**1–27**) in DMSO (Sigma) were prepared over the range 3.9–250 μg mL^{-1}. Testing was performed in 96-well round-bottom microtitration plates. Two growth controls, using test medium alone and with 2.0% (v/v) DMSO, and a sterility control (drug-free medium only and medium with the xanthones) were included in all assays. The plates were incubated at 35 °C (for *C. albicans*, *C. glabrata*, *C. neoformans,* and *A. fumigatus*) or 25 °C (for dermatophytes) during 48 h (*Candida* spp.), 72 h (*C. neoformans* and *A. fumigatus*), and 7 days (dermatophytes). MICs were determined as the lowest concentrations capable of substantially inhibiting fungal growth in comparison with controls. All results are from three independent and concordant experiments, performed in duplicate. In addition, the antifungal compound fluconazole was used as the standard antifungal drug. Twofold serial dilutions ranging from 3.9 to 250 μg mL^{-1} were used. Quality control determinations of the fluconazole MICs were ensured by testing *C. parapsilosis* ATCC 90018. The results obtained were within the recommended limits (data not shown).

Sterol extraction

Cell suspensions of the different strains of fungi were prepared on distilled water from recent cultures on SDA with chloramphenicol, at a density of 10^6 CFU mL^{-1}. Fifty microliters of these cell suspensions was diluted in RPMI-1640 medium, yielding a final inoculum size of 10^3 CFU mL^{-1}. Several twofold dilutions of xanthones **10** and **13** and fluconazole were prepared and added to the cell suspensions. Cultures were incubated with shaking at 35/C (for *C. albicans*, *C. neoformans,* and *A. fumigatus*) or 25 °C (for dermatophytes) during 48 h (*C. albicans*), 72 h (*C. neoformans* and *A. fumigatus*), and 7 days (dermatophytes). A quantification of ergosterol amount was performed after incubation with xanthones **10** and **13** or fluconazole and without as control, at different concentrations.

The sterol extraction was adapted from Arthington-Skaggs *et al.* (56). Briefly, fungal cells were harvested by centrifugation (*C. albicans* and *C. neoformans*) at 980× *g* for 5 min or by filtration (*A. fumigatus* and *T. mentagrophytes*), and the pellets were washed twice with sterile distilled water. The wet pellet weight was adjusted to 0.100 g, and 3 mL of 25% alcoholic potassium hydroxide solution was added, followed by a vigorous agitation in a vortex for 1 min. Cell suspensions were incubated in a water bath at 85 °C during 60 min. Following incubation, tubes were removed and left to cool at room temperature. Sterols were then extracted by addition of 1 mL of sterile distilled water and 3 mL of *n*-heptane (Romil Chemicals, Leics., England) to each tube, followed by a vigorous vortex agitation for 3 min. The organic phase (*n*-heptane) was then transferred to a clean glass tube, and the *n*-heptane was evaporated to dryness, under a nitrogen stream. The extracted sterols were redissolved in 1 mL of dichloromethane (Merck) prior to HPLC-UV analysis.

Sterol analysis

Ergosterol was analyzed by HPLC with ultraviolet (UV) detection based on the method proposed by Peacock and Goosey (57). The chromatography was performed with a system consisting of two high-pressure pumps (Jasco 880-PU intelligent HPLC pump I), a manual injector (rheodyne 7125), and a spectrophotometer detector (Jasco 875-UV intelligent UV/Vis). The column was stainless steel (250 × 4.6 mm), packed with Hypersil silica 3 μm (Hichrom). A guard column (10 × 4.6 mm) filled with Hypersil silica 5 μm was used. The mobile phase was a solution of methanol (Merck) in dichloromethane 0.025% (v/v), and the flow rate was 1.0 mL min^{-1} with an injection volume of 100 μL and a race time of about

20 min. Detection was performed at 245 nm. Methanol, dichloromethane, and *n*-heptane were HPLC grade. Ergosterol (**E**) and lanosterol (**L**) (Sigma) were analytical grade.

Validation procedure

The method was validated by ICH described parameters (58). Linearity was evaluated in triplicates of at least five calibration standard solutions of ergosterol. The regression line was calculated as $y = a + bx$, by plotting analyte concentration (x), namely ergosterol (**E**) (expressed as mg/100 g wet fungal cells), versus average peak area (y) (expressed as mAU). The calibration curves were obtained using the linear least squares regression procedure. The RSD (relative standard deviation) values for the response factors of three experiments were calculated. For intraday precision, three concentrations of the extracted sterols were analyzed within 24 h and injected at least five times. For intermediate precision, samples from three concentrations of the extracted sterols were analyzed on five consecutive days assayed in quintuplicate. Precision was expressed as RSD. The limits of detection (LOD) and quantification (LOQ) were evaluated by analyzing ten blank samples. The specificity of the analytical method in this study was determined by the analysis of two other sterols involved in the ergosterol pathway: lanosterol (early sterol pathway intermediate) and 24 (**28**) dehydroergosterol (late sterol pathway intermediate) which absorb in the same λ values. Under the applied conditions, no interference from these mycosterols was observed at the retention time for ergosterol. Recovery studies were performed using the minimum, maximum, and an intermediary concentration; accuracy was determined by spiking three known concentrations of ergosterol (9.6, 96, 192 μg mL^{-1}) obtained from the test samples.

Results

Chemistry

Among the investigated xanthones (**1–27**), four are from higher plants of the Clusiacea family: 2-hydroxy-1-methoxyxanthone (**24**) (41), 1,7-dihydroxyxanthone (euxanthone, **25**), 2-hydroxy-1,8-dimethoxyxanthone (**26**) (52), and 1,2,8-trimethoxyxanthone (**27**) from *Calophyllum teysmannii* var. *inophylloide* (52). The majority of the oxygenated xanthones (**2–22**) were obtained by classical methods *via* benzophenone or a biphenyl ether intermediates (59), and their syntheses are described elsewhere (38,49,53). In this study, a one pot synthesis for 3,6-dihydroxyxanthone (**23**) was performed through a dehydrative cyclization of the commercially available 2,2′,4,4′-tetrahydroxybenzophenone (**28**, Figure 2). This method is preferred to the previously described procedures (60,61) because no purification is needed to furnish compound **23** in quantitative yields.

Antifungal activity

To investigate the antifungal activity of simple oxygenated xanthones, 8 monosubstituted, 16 disubstituted, and 2 trisubstituted xanthones along with the xanthone (**1**) were evaluated for their growth inhibitory effect against three yeasts (*C. albicans*, *C. glabata*, *C. neoformans*), a non-dermatophyte filamentous fungi (*A. fumigatus*), and five dermatophyte filamentous fungi (*M. gypseum*, *M. canis*, *E. floccosum*, *T. mentagrophytes*, and *T. rubrum*). The results (oxygenated xanthones **1**–**27**) are presented in Table 1.

Xanthone (**1**) did not inhibit the growth of the examined strains even when tested at concentrations higher than 250 μg mL^{-1}. However, introduction of oxygenated substituents on the xanthone nucleus has led to the appearance of an inhibitory effect (MIC<250 μg mL^{-1}). Xanthones **3–6**,**10–12**,**16**,**19**,**23**,**25–27** were found to inhibit the growth of the fungal strains tested (Table 1).

Although the majority of these xanthones did not show a very strong activity, some of them (**3–5**,**10**,**12**) exhibited striking inhibitory effects with MIC values <10 μg mL^{-1}, while compounds **1–2,7–9,13–15,17,18,20–22,24** were found to be inactive against all the tested organisms (MIC>250 μg mL^{-1}).

From the results shown in Table 1, it was found that except for 1,7-dihydroxyxanthone (**25**) that was selective to *E. floccosum* (MIC = 15.6 μg mL^{-1}) and for 3-hydroxy-4-methoxyxanthone (**16**), all the other active xanthones (**3–6**,**10–12**,**19**,**23**,**26–27**) inhibited the growth of the five dermatophyte filamentous fungi, with MIC values ranging from 7.8 to 250 μg mL^{-1}. They were, in general, more active against *E. floccosum* (7.8–125 μg mL^{-1}) and less active against *T. rubrum* (15.2–250 μg mL^{-1}). 4-Hydroxy-(**5**) and 1,2-dihydroxyxanthone (**10**) also inhibited *C. neoformans* (MIC = 31.3 μg mL^{-1}) and *A. fumigatus* (MIC = 62.5 and 31.3 μg mL^{-1}, respectively); however, only compound **10** was active against *Candida* species (MIC = 31.3 μg mL^{-1}).

Sterol content

Sterols were extracted from cultures of four fungal strains (*C. albicans*, *C. neoformans*, *A. fumigatus*, and *T. mentagrophytes*) and analyzed by normal-phase HPLC. Separations of sterols were optimized for the described experimental conditions used in the validation procedure. The assay was successfully validated with respect to

Figure 2: Synthesis of 3,6-dihydroxyxanthone (**23**) from 2,2′,4,4′-tetrahydroxybenzophenone (**28**).

Table 1: Antifungal activity (MIC [a], μg mL^{-1}) of 27 xanthones and fluconazole against *Candida*, *Cryptococcus*, *Aspergillus*, and dermatophyte strains[b]

No	Xanthone	*C. albicans*	*C. glabrata*	*C. neoformans*	*A. fumigatus*	*M. gypseum*	*M. canis*	*E. floccosum*	*T. mentagrophytes*	*T. rubrum*
1	Xanthone	>250	>250	>250	>250	>250	>250	>250	>250	>250
2	1-hydroxy	>250	>250	>250	>250	>250	>250	>250	>250	>250
3	2-hydroxy	>250	>250	>250	>250	**15.6**	**15.6**	**7.8**	**7.8**	**15.6**
4	3-hydroxy	>250	>250	>250	>250	**15.6**	**250**	**7.8**	**7.8**	250
5	4-hydroxy	>250	>250	**31.3**	**62.5**	**15.6**	**15.6**	**7.8**	**15.6**	**31.3**
6	1-methoxy	>250	>250	>250	>250	**31.3**	**31.3**	**31.3**	**31.3**	**62.5**
7	2-methoxy	>250	>250	>250	>250	>250	>250	>250	>250	>250
8	3-methoxy	>250	>250	>250	>250	>250	>250	>250	>250	>250
9	4-methoxy	>250	>250	>250	>250	>250	>250	>250	>250	>250
10	1,2-dihydroxy	**31.3**	**31.3**	**31.3**	**31.3**	**7.8**	**15.6**	**15.6**	**7.8**	**31.3**
11	2,3-dihydroxy	>250	>250	>250	>250	**31.3**	**31.3**	**15.6**	**31.3**	**31.3**
12	3,4-dihydroxy	>250	>250	>250	>250	**15.6**	**15.6**	**7.8**	**31.3**	**31.3**
13	1,2-dimethoxy	>250	>250	>250	>250	>250	>250	250	>250	>250
14	2,3-dimethoxy	>250	>250	>250	>250	>250	>250	>250	>250	>250
15	3,4-dimethoxy	>250	>250	>250	>250	>250	>250	>250	>250	>250
16	3-hydroxy -4-methoxy	>250	>250	>250	>250	**125**	>250	**31.3**	**125**	>250
17	4-hydroxy -3-methoxy	>250	>250	>250	>250	>250	>250	>250	>250	>250
18	3,5-dihydroxy	>250	>250	>250	>250	>250	>250	>250	>250	>250
19	3,5-dimethoxy	>250	>250	>250	>250	**125**	**125**	**62.5**	**125**	**125**
20	3-hydroxy-5-methoxy	>250	>250	>250	>250	>250	>250	>250	>250	>250
21	1,3-dimethoxy	>250	>250	>250	>250	>250	>250	>250	>250	>250
22	2,7-dimethoxy	>250	>250	>250	>250	>250	>250	>250	>250	>250
23	3,6-dihydroxy	>250	>250	>250	>250	**125**[d]	250[d]	250[d]	250[d]	250[d]
24	2-hydroxy-1-methoxy[c]	>250	>250	>250	>250	>250	>250	>250	>250	>250
25	1,7-dihydroxy[c]	>250	>250	>250	>250	>250	>250	**15.6**	>250	>250
26	2-hydroxy-1,8-dimethoxy[c]	>250	>250	>250	>250	**125**	**125**	**125**	**125**	**125**
27	1,2,8-trimethoxy[c]	>250	>250	>250	>250	**125**	**125**	**62.5**	**62.5–125**	**125**
——	fluconazole	64	64	32	>128	4	8	2	2	0.5

[a]Results are expressed as MIC (minimal inhibitory concentration) in μg mL^{-1} and show means of three independent observations made in duplicate.
[b]Isolate sources: *C. glabrata* D10R, from recurrent cases of oral candidosis; *Cryptococcus neoformans* PH1, from cerebrospinal fluid; *Microsporum canis* FF1, *M. gypseum* FF3, *Trichophyton mentagrophytes* FF7, *T. rubrum* FF5, and *Epidermophyton floccosum* FF9, all isolated from nails and skin and ATCC (American Type Culture Collection) type strains (*C. albicans* ATCC 10231 and *Aspergillus fumigatus* ATCC 46645).
[c]Isolated from natural sources.
[d]Showed a slight inhibitory effect at this concentration against these strains; MICs were not determined.

specificity, linearity ($y = -54.3 + 16.0x$; $R = 0.998$; relative standard deviation RSD $\leq$ 3.5%), range, precision (RSD $\leq$ 7.5%), and limits of detection (LOD = 0.71 μM) and quantification (LOQ = 2.15 μM), according to ICH guidelines (58). Recovery data were within the range of 61.2–75.9%.

Ergosterol (**E**, t_R 17.8–18.2 min) and lanosterol (**L**, t_R 10.5–10.7 min) were detected at their maxima absorption (245 nm), and compounds were identified by coinjection with real standards. Both compounds were well separated, without interferences, in all samples. This method was applied to the analysis of the ergosterol content from fungal cells treated with different concentrations of 1,2-dihydroxyxanthone (**10**), which is active against all the tested fungi, and its dimethoxylated analog 1,2-dimethoxyxanthone (**13**), which is inactive against all tested fungi and fluconazole, a well-known inhibitor of ergosterol biosynthesis. A model of chromatograms is presented in Figure 3, and Table 2 displays the results obtained for controls, compound **10** and fluconazole. For compound **13**, a relationship between xanthone concentration and amount of ergosterol was not observed (data not shown). For 1,2-dihydroxyxanthone (**10**), the relationship between concentration and amount of ergosterol is visible for all the fungi studied.

The effect of 1,2-dihydroxyxanthone (**10**) on ergosterol level varies with its concentrations and the tested organisms. In *C. albicans* and *C. neoformans,* the lowest concentration (3.9 μg mL^{-1}) of 1,2-dihydroxyxanthone (**10**) does not appear to show a difference or, if anything, a slightly higher level of ergosterol, while at higher concentrations (7.8 and 15.6 μg mL^{-1}), ergosterol levels appear to be lower than those of control. On the contrary, ergosterol levels in *A. fumigatus* were higher than those of the control for all concentrations (3.9, 7.8, and 15.6 μg mL^{-1}) of 1,2-dihydroxyxanthone (**10**). On the other hand, *T. mentagrophytes* was found to be more sensitive to 1,2-dihydroxyxanthone (**10**), which was able to inhibit ergos-

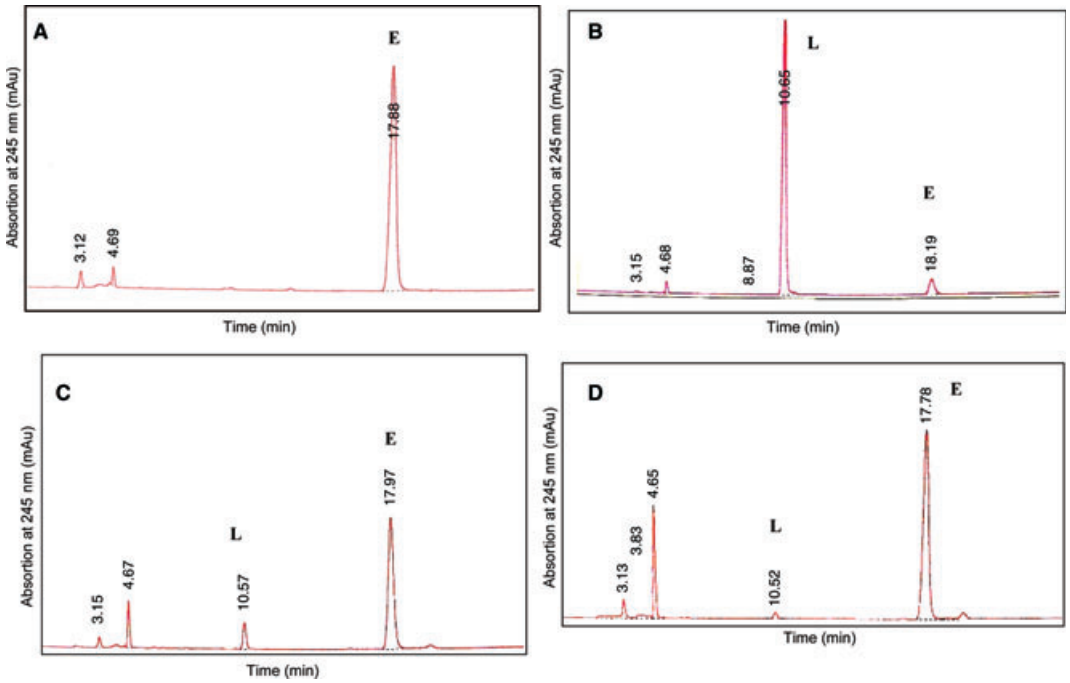

Figure 3: HPLC chromatograms obtained from extracts of cells of *T. mentagrophytes* A) untreated or B) treated with 3.9 μg mL^{-1}, C) 1.9 μg mL^{-1}, and D) 0.98 μg mL^{-1} of 1,2-dihydroxyxanthone (**10**). L = lanosterol, E = ergosterol. Conditions: silica: methanol in dichloromethane 0.025% (v/v), 1.0 mL min^{-1}.

terol synthesis at a concentration of 3.9 μg mL^{-1} (Table 2). At lower concentrations (1.95 and 0.98 μg mL^{-1}), the levels of ergosterol were found to be higher than those of the control.

For fluconazole, the relationship between concentration and amount of ergosterol is visible for all the fungi under study. All concentrations (0.5, 1.0, 2.0 μg mL^{-1}) of fluconazole gave lower ergosterol levels than those of the controls in *C. albicans*, while this happened only at the concentrations of 1.0 and 2.0 μg mL^{-1} in *C. neoformans* (Table 2). Only at a concentration as high as 128 μg mL^{-1} of fluconazole, the amount of ergosterol was lower than that in the control for *A. fumigatus*. *T. mentagrophytes* was most sensitive to fluconazole as well, showing lower ergosterol contents than in the control when incubated with fluconazole at the concentrations of 0.125 and 0.25 μg mL^{-1}.

Discussion

Plants present a unique pool of compounds in the search for new antifungal lead structures because of the variety and chemical complexity of their constituents. Previous studies have reported some xanthone derivatives as remarkable antifungal agents. The antifungal profile of the described xanthones suggests that, in the majority of cases, hydroxyl groups are important for activity (8,13,62,63). However, because of biosynthesis limitations of these natural products, the pattern of oxygenation is most frequently restricted to positions 1,3,5,6 for simple oxygenated and prenylated xanthones and to 1,4,8 for polycyclic and dehydroxanthones. These facts, allied with our experience in this class of compounds, have prompted us to investigate a series of simple oxygenated xanthones for their potential antifungal properties.

All dermatophytes investigated were found to be sensitive to fluconazole (which is in accordance with CLSI guidelines), and results revealed that, among the 27 xanthones tested, the number of xanthones capable of inhibiting their growth is much higher than that for the non-dermatophytes group (Table 1). Among the investigated xanthones, 1,2-dihydroxyxanthone (**10**) showed a broad spectrum of activity, being active against all the nine fungal strains tested.

Interestingly, with the exception of compounds **5** and **10,** some selectivity toward dermatophyte filamentous fungi was observed, with compounds **3,4,6**,**11,12**,**16**,**19**,**23**,**25–27** showing no activity against *Candida* species, *C. neoformans,* or *A. fumigatus* even at the maximum concentrations tested (250 μg mL^{-1}). In contrast, compounds **5** and **10** were active in the same range of concentrations as fluconazole to *C. neoformans* and were found to inhibit the growth of *A. fumigatus* (MIC = 62.5 and 31.3 μg mL^{-1}, respectively), which displays the lowest susceptibility to fluconazole (MIC > 128 μg mL^{-1}). It is also interesting to point out that xanthone (**1**), 2-hydroxy-1-methoxyxanthone (**24**), and 1,7-dihydroxyxanthone (**25**) did not show any growth inhibitory activity against *C. albicans*, *C. glabrata*, or *C. neoformans* (Table 1), these results being in agreement with those reported previously for these compounds (8,10,12,20). Although compound **24** has been previously reported as responsible for the antifungal activity of the extract of *Kielmeyera coriacea* against *Cladosporium cucumerinum* (12), it showed no antifungal activity against all the nine fungal strains

Table 2: Ergosterol content (mg/100 g wet fungal cells) from fungal cells treated with different concentrations of 1,2-dihydroxyxanthone (10) and fluconazole

Fungi strains	Compound	Compound concentration (μg mL^{-1})	Ergosterol concentration ± SD[a]
Candida albicans	Control	0.0	25.80 ± 7.31
	10	15.6	15.94 ± 4.61
		7.8	20.34 ± 2.83
		3.9	26.60 ± 2.17
	Fluconazole	2.0	5.88 ± 3.04
		1.0	7.32 ± 2.56
		0.5	15.86 ± 2.32
Cryptococcus neoformans	Control	0.0	23.22 ± 0.24
	10	15.6	20.00 ± 5.58
		7.8	22.96 ± 3.79
		3.9	29.58 ± 8.30
	Fluconazole	2.0	15.24 ± 1.77
		1.0	21.48 ± 2.55
		0.5	27.39 ± 1.01
Aspergillus fumigatus	Control	0.0	39.30 ± 7.54
	10	15.6	39.97 ± 7.61
		7.8	43.41 ± 10.85
		3.9	54.42 ± 4.23
	Fluconazole	128	29.02 ± 6.13
		32	68.96 ± 7.80
		8	70.59 ± 6.83
Trichophyton mentagrophytes	Control	0.0	43.44 ± 8.16
	10	3.9	16.84 ± 0.61
		1.95	43.79 ± 7.25
		0.98	56.55 ± 15.28
	Fluconazole	0.25	30.23 ± 1.25
		0.125	37.24 ± 3.85
		0.063	79.07 ± 4.52

[a]Results are presented as mg/100 g wet fungal cells and show means of three independent analyses ± SD.

investigated here (Table 1). Also, 2-hydroxyxanthone (**3**) was previously described to inhibit *A. fumigatus* with a MIC value of 31 μg mL^{-1} (64). Various factors may contribute to this discrepancy, namely different pH conditions (65).

Interestingly, Gopalakrishnan *et al.* (8) have suggested, from the correlation of the antifungal activity profiles and the structures of xanthone (**1**) and euxanthone (**25**), that the presence of hydroxyl groups in rings A and B was important for the antifungal activity. Furthermore, the nature of the substituents seems to influence the growth inhibitory effect of xanthones (Table 1). Thus, most mono- (**7–9**) and dimethoxylated (**13–15**,**21,22**) xanthones were found to be inactive against the test fungi, while 3,5-dimethoxyxanthone (**19**) and 1,2,8-trimethoxyxanthone (**27**) showed only a mild inhibitory effect against dermatophytes. Oddly enough, the only active methoxyxanthone was 1-methoxyxanthone (**6**), possibly because of the close proximity of the methoxyl group with the carbonyl group. In this study, antifungal activity profiles of simple oxygenated xanthones and the correlation with their structures also suggest that free hydroxyl groups in one ring of the xanthone nucleus are important for optimal activity, as observed for monohydroxyxanthones **3–5** and for dihydroxyxanthones **10–12**. The quinone substructure has been frequently characterized in compounds having strong anticandidal activity. A keto-enol tautomerism-based formation of a reactive quinone methide intermediate was previously described to explain the antifungal activity of flavonoids (65). In a similar way,

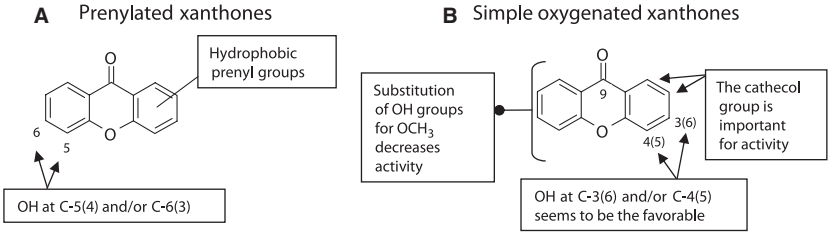

Figure 4: Qualitative structure–activity relationship for antifungal activity most favorable molecular substitutions for (A) prenylated (7,13) and (B) simple oxygenated xanthones.

the ability of hydroxyxathones to form quinone substructures (66,67) may explain the fact that the highest effect was observed for compounds **3–5** and **10–12**. These findings support the data from the recently reported antifungal activity of prenylated xanthones isolated from *Cratoxylum cochinchinense*, in which only compounds with a catechol moiety exhibited strong activity (11). The absence of the effect observed for 1-hydroxyxanthone (**2**) can be justified by the formation of hydrogen bonding between the carbonyl and hydroxyl groups at C-1. In contrast, hydroxylation in both rings (A and B) of the xanthone nucleus does not favor the antifungal activity, as can be observed for compounds **18, 23**, and **25**. Additionally, it can be affirmed that monomethylation of the catechol moiety (in position 2 for **24** with respect to **10** and in position 3 for **17** with respect to **12**) could be the reason for the loss of the antifungal activity. However, this effect was less pronounced for position 4 (from the observation of **12** and **16**). Opinions diverge concerning the effect of hydroxyl groups on the antifungal activity of xanthones. While some authors have stated that only hydroxyl groups in the xanthone rings could increase the antifungal activity (13), further suggesting that hydroxyl groups in the side chains did not seem to affect the activity, others (63) reported as likely that antifungal xanthones from plants required three or four hydroxyl groups, in which one or two of them must be at C-5 (C-4) and/or C-6 (C-3), and a hydrophobic group must be on one of the aromatic rings as illustrated in Figure 4A. Herein, some relationships previously established for anticandidal flavonoids (65) and prenylated xanthones can be extrapolated to xanthones (Figure 4B): a hydroxyl group in position 3 and/or 4 seems to be favorable and a keto group must be present in position 9. Additionally, a catechol group is important for activity, and substitution of hydroxyl by methoxyl groups is associated with the decrease in the activity.

Not many xanthone-derived compounds have been examined for their mechanism of action in detail. In the case of dehydroxanthones, the biochemical target of these natural products has been identified as the fungal polyadenosine polymerase (33). The putative mechanism of prenylated flavonoids, antifungal small molecules related to xanthones, pointed the cell membrane as a possible target (68). Nonetheless, the most often described target of antifungal drugs is ergosterol biosynthesis (65). Because of the antifungal profiles of fluconazole and xanthones **3–6**,**10–12**,**16**,**19**,**23**,**25–27** (Table 1), it can be hypothesized that xanthones act, like azoles, by the inhibition of ergosterol biosynthesis. The results obtained from the ergosterol determination (Table 2) seem to corroborate this hypothesis. As expected, fluconazole and 1,2-dihydroxyxanthone (**10**) showed a relationship between their concentrations and the amount of ergosterol detected, i.e., with the increase in concentration of these antifungal compounds, a decrease in the ergosterol content was observed in the investigated strains (Table 2). As expected, contrary to 1,2-dihydroxyxanthone (**10**) and fluconazole, 1,2-dimethoxyxanthone (**13**), an inactive compound, did not affect ergosterol biosynthesis.

If 1,2-dihydroxyxanthone (**10**) acts as an inhibitor of ergosterol biosynthesis, it seems reasonable to assume that other reported simple oxygenated xanthones (on account of their structural similarity with compound **10**) can act by the same mechanism. Additionally, the metal ion-chelating abilities of catecholic xanthones (67) can influence their antifungal inhibitory effect. In fact, inhibitors of fungal iron acquisition may provide attractive antifungal therapies (69,70), and this hypothesis deserves to be explored in the future.

Conclusion

In conclusion, the investigation into the antifungal activity of 27 simple oxygenated xanthones has led us to obtain some interesting structure–activity relationships for this class of compounds. The simple, rapid, and efficient HPLC-UV method for the identification and quantification of fungal sterols in whole cells of *Candida*, *Cryptococcus*, *Aspergillus,* and dermatophyte strains has been developed and validated, and this method can be used as a tool to evaluate the antifungal activity of the compounds that inhibit the ergosterol biosynthesis. 1,2-Dihydroxyxanthone (**10**) was identified as a valuable scaffold for further development of more complex and diverse xanthone derivatives for broad-spectrum antifungal activity.

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