Synthesis and biological evaluation of substituted α- and β-2,3-dihydrofuran naphthoquinones as potent anticandidal agents†

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We report herein, the synthesis and antifungal activity of substituted α- and β-dihydrofuran naphthoquinones. These compounds were prepared from readily available lawsone and olefins in the presence of cerium(IV) ammonium nitrate (CAN) and were then evaluated against the following six strains of *Candida* (*C.*): *C. albicans*, *C. krusei*, *C. parapsilosis*, *C. kefyr*, *C. tropicalis and C. dubliniensis*. In addition to exhibiting low cytotoxicity, the furan naphthoquinones proved to be active against these fungi, indicating that they are important scaffolds and potential novel antifungal agents.

1. Introduction

The recent rise of fungal infections may be primarily attributed to the increasing numbers of immunocompromised patients, including those with human immunodeficiency virus infection, cancer patients undergoing chemotherapy and organ transplant recipients taking immunosuppressive drugs.[1] Fluconazole and Itraconazole are the most commonly used therapeutics for fungal infections in clinical settings; however, they have major weaknesses regarding their spectra, potency, safety and pharmacokinetic properties. Additionally, the emergence of strains that are resistant to existing antifungal agents, such as *Candida*, is becoming a significant problem.[2,3] Thus, the development of novel, effective antifungal agents is strongly needed.[4–6]

Naphthoquinones represent an important class of biologically active molecules that are widespread in nature.[7] Interest in these substances has been intensified in recent years due to their wide range of biological activities. Many natural and synthetic naphthoquinones are known to be potent antitumor,[8,9] molluscicidal,[10] leischmanicidal,[11] anti-inflammatory,[12] tripanocidal,[13] antibacterial[14] and antitubercular[15] agents. A number of studies have also demonstrated that substituted naphthoquinone derivatives show a particularly marked activity against fungi.[16–22] Recently, our group reported the syntheses of novel dihydrofuran naphthoquinone compounds that show great potential in pharmacological applications.[23,24]

The present study was focused on the synthesis of α- and β-2,3-dihydrofuran naphthoquinones and their evaluation against six strains of *Candida*, isolated from the oral cavity of patients with removable dentures, and an ATCC (American Type Culture Collection) reference strain, *C. albicans*.

2. Material and methods

2.1. Chemistry

The preparation of α- and β-2,3-dihydrofuran naphthoquinones, **1a–i** and **2a–i**, respectively, were carried out in one step, following an improved synthetic protocol that was previously reported.[25,22] Briefly, the furan naphthoquinones were obtained by oxidative [3 + 2] cycloaddition of 2-hydroxy-1,4-naphthoquinone (Lawsone, **3**) to the alkene, mediated by cerium(IV) ammonium nitrate (CAN) (Scheme 1). All the compounds were obtained in good yields (10–86% of isolated α and β products) and were fully characterized by proton and carbon nuclear magnetic resonance spectroscopy (^{1}H NMR and ^{13}C NMR, respectively), infrared spectroscopy (IR) and elemental analysis.

2.2 *In vitro* antifungal activities

The antifungal activities of **1a–i** and **2a–i** were assessed against *C. albicans*, *C. krusei*, *C. parapsilosis*, *C. kefyr*, *C. tropicalis and C. dubliniensis*, isolated from the oral cavity of patients with removable dentures, by a diffusion technique and minimum inhibitory concentration (MIC) assay. Activity against an ATCC reference strain of *C. albicans* (90028) was also assessed to ensure result reproducibility.[26]

Diffusion technique. The susceptibility of synthetic furan naphthoquinones was tested initially by simple diffusion from the hole in the culture medium (hole plate). All fungi were grown in Sabouraud dextrose agar (SDA) medium. The procedural kit provided a standard yeast suspension containing 1×10^6 –5×10^6 cells per mL. After cell seeding, four holes, 6 mm in diameter, were introduced with a sterile Pasteur pipette. In three of four holes, 20 μL of each potential antifungal agent with a concentration of 250 μg mL^{-1} was added, and 20 μL of DMSO was delivered to the remaining hole. The plates were then incubated for 24 h at 37 °C. After incubation, the inhibition zone of fungal

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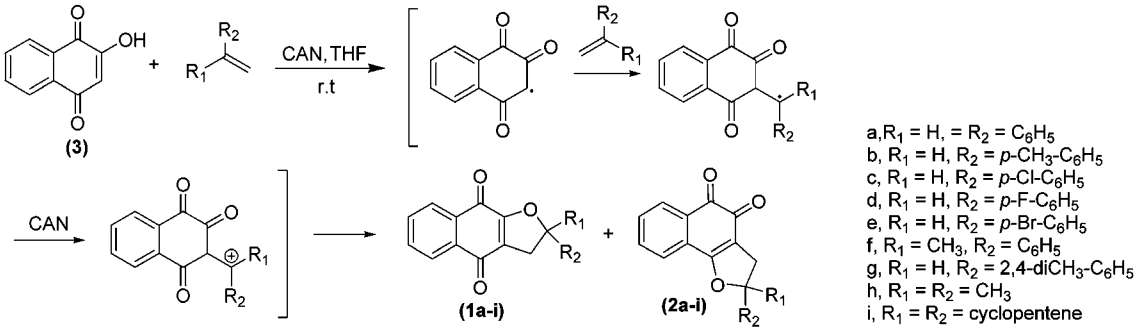

Scheme 1 Synthetic route used for the preparation of α- and β-furan naphthoquinones, **1a–i** and **2a–i**, respectively.

growth was measured. We used antifungal drugs, fluconazole and itraconazole for standard comparison of the activity. The control hole containing DMSO was used as a reference in the assay. All determinations were performed in triplicate, and the DMSO zone was subtracted from the data output.

Minimum inhibitory concentration (MIC) assay. In this process, the MIC of α- and β-furan naphthoquinones, **1a–i** and **2a–i**, were obtained according to the standard microbroth dilution. Briefly, the MIC assay was performed in flat-bottomed, 96-well tissue culture plate in SDA medium. Compounds **1a–i** and **2a–i** were dissolved in DMSO. These plates were incubated at 35 °C for 24 h before being read. Fluconazole and Itraconazole were used as antifungal standards, and all determinations were performed in triplicate. MIC was defined as the lowest concentration preventing visible fungal growth.

3. Results and discussion

The α- and β-2,3-dihydropyran naphthoquinones were obtained in good chemical yields (10–86% of isolated α and β products) with a high degree of purity. When a solution of 2-hidroxy-1,4-naphthoquinone (**3**) and alkene in THF (1 : 1) was treated with a solution of CAN (2.3 eq.) in THF (room temperature, 3 h), two products, α- and β-2,3-dihydropyran naphthoquinone were generated (Scheme 1). It is noteworthy that all the products were formed regioselectively, with respect to the double bond of the alkene. Products were isolated by silica gel column chromatography (Table 1).

All compound structures were confirmed by IR spectral data, 1D and 2D NMR techniques and by elemental analysis. The compounds **1a–c**, **1f**, **1h**, **1i**, **2a–c**, **2f**, **2h** and **2i**[25,27] have been previously described in literature, and our spectroscopic data are consistent with those reported. In general, the IR spectra showed the disappearance of an intense absorption band, referring to the stretching vibrations of O–H from the starting material to the products. All spectra displayed strong carbonyl absorption between 1670–1702 cm^{-1}. Additionally, all proton signals (^1H NMR spectra) were assigned to peaks on the basis of their chemical shifts, multiplicities and coupling constants. For the α-derivatives, the ^1H NMR spectra primarily displayed the signal of the hydrogen bonded to the *ortho* carbon of the chromogenic ring as a doublet of doublets between 5.15–6.30 ppm. The hydrogen signals of the CH_2 located in the furan ring were assigned in the region of 2.00 to 3.67 ppm as a doublet of doublets. The assignment of α or β-furan naphthoquinones were established based on the analysis of the aromatic hydrogen signal region. The aromatic hydrogens in β-dihydrofuran naphthoquinones are differentiated into four signals, while in the α-dihydrofuran naphthoquinones, there are only two ^1H signals due to the symmetric aromatic ring. In general, the ^{13}C NMR spectra of naphthoquinones, **1a–i** and **2a–i** were easily distinguished by the signals between 79.2–80.0 ppm and 81.0–82.0 ppm, respectively, due to substituted carbon of the chromogenic ring.

Table 1 Yields of **1a–i** and **2a–i**

Entry	R1	R2	1 (%)	2 (%)
1	H	C_6H_5	**1a** (70)	**2a** (28)
2	H	*p*-CH_3–C_6H_5	**1b** (75)	**2b** (13)
3	H	*p*-Cl–C_6H_5	**1c** (85)	**2c** (12)
4	H	*p*-F–C_6H_5	**1d** (65)	**2d** (12)
5	H	*p*-Br–C_6H_5	**1e** (45)	**2e** (10)
6	CH_3	C_6H_5	**1f** (60)	**2f** (32)
7	H	2,4-diCH_3-C_6H_5	**1g** (75)	**2g** (20)
8	CH_3	CH_3	**1h** (86)	**2h** (12)
9	—	Cyclopentadiene	**1i** (15)	**2i** (30)

The first technique employed to evaluate the antifungal activity of compounds **1a–i** and **2a–i** was diffusion in solid medium through a hole. In these studies, substances that influence halo formation of 9–14 mm, 14–17 mm or >17 mm are considered to be moderate, active, or very active antifungal agents, respectively. In this regard, all of our naphthoquinones have lower activity than itraconazole and some of them have higher activity than fluconazole. Compounds **1a**, **1h** and **1i** demonstrate significant inhibitory activity on *C. albicans*, with inhibitory zones of 21.26, 16.97 and 16.62 mm, respectively, as shown in Table 2. Compound **1h**, **1i** and **2i** also show good activity profile against *C. dubliniensis*.[28]

The second technique used to determine the antifungal activity of furan naphthoquinones was a microdilution quantitative assay in which fungi cultures were exposed to antifungal agents for 24 h. The percentage of growth reduction was then measured by visual inspection and the analysis of the turbidity of the samples in different concentrations. The standard developed by the National Committee for Clinical Laboratory Standards (NCCLS)[29] defines the MIC as the lowest antimicrobial agent concentration that inhibits visible growth of a microorganism in the midst of a dilution made in liquid medium. The MIC values of all substances tested, as well as the major antifungal standards are listed in Table 3. The results revealed that substituted α-furan

Table 2 Antifungal activity of compounds **1a–i** and **2a–i** as determined by diffusion showing growth inhibition zones (mm). Mean ± S.D., in triplicate

Compound	*C. albicans*	*C. tropicalis*	*C. kefyr*	*C. parapsilosis*	*C. krusei*	*C. dubliniensis*
1a	16.97 ± 4.02	7.7 ± 2.4	1.96 ± 3.4	6.88 ± 1.15	8.29 ± 2.55	4.3 ± 1.00
1b	10.43 ± 2.62	7.28 ± 2.18	4.27 ± 3.71	0	4.94 ± 1.15	2.75 ± 0.92
1c	1.5 ± 1.10	2.49 ± 0.07	5. 27 ± 4.66	0	6.14 ± 1.00	0
1d	10.34 ± 2.95	3.31 ± 1.35	7.28 ± 1.14	3. 86 ± 4.59	9.12 ± 2.32	2.66 ± 1.26
1e	1.39 ± 0.40	0	0	9.18 ± 2.10	7.50 ± 2.36	0.49 ± 0.38
1f	16.14 ± 4.25	3.83 ± 2.14	7.42 ± 0.98	0	7.05 ± 1.32	4.63 ± 1.20
1g	1.38 ± 1.02	6.42 ± 1.84	0	0	9.87 ± 1.88	2.02 ± 0.33
1h	21.26 ± 0.87	5.63 ± 2.76	18.44 ± 0.77	9.29 ± 0.62	14.63 ± 1.42	15.23 ± 1.45
1i	16.62 ± 1.99	7.8 ± 1.77	9.3 ± 1.24	0	10.76 ± 3.50	16.60 ± 0.59
2a	7.37 ± 1.74	5.7 ± 0.65	10.84 ± 3.14	7.22 ± 1.13	11.99 ± 2.43	11.95 ± 0.48
2b	0.39 ± 0.78	2.26 ± 2.08	0	0	5.77 ± 0.53	6.44 ± 1.02
2c	1.5 ± 1.3	2.21 ± 0.83	1.96 ± 3.4	6.11 ± 0.82	5.65 ± 1.41	9.01 ± 0.10
2d	1.37 ± 0.47	8.59 ± 1.53	8.96 ± 1.06	8.12 ± 1.23	9.8 ± 1.36	1.70 ± 1.28
2e	6.17 ± 1.64	0	8.68 ± 6.77	7.75 ± 0.51	11.28 ± 2.59	9.23 ± 0.78
2f	8.41 ± 0.4	3.48 ± 0.79	10.08 ± 0.54	7.34 ± 1.01	8.52 ± 1.92	12.88 ± 0.55
2g	2.93 ± 0.71	7.51 ± 2.84	9.28 ± 0.59	9 ± 0.05	10.83 ± 1.91	4.19 ± 0.05
2h	1.64 ± 0.71	4.78 ± 0.47	8.27 ± 1.16	7.41 ± 1.17	9.01 ± 2.54	0.10 ± 0.21
2i	5.85 ± 0.60	7.19 ± 1.57	10.70 ± 0.86	6.14 ± 0.80	8.80 ± 1.40	18.53 ± 1.36
Itraconazole	23.06 ± 0	23.63 ± 2.03	30.88 ± 2.20	26.01 ± 0.93	30.34 ± 4.32	19.45 ± 1.21
Fluconazole	12.99 ± 2.75	14.97 ± 3.56	21.23 ± 0.83	10.38 ± 0.95	9.64 ± 0.91	12.84 ± 1.05

Table 3 Minimum inhibitory concentration (MIC, μM) data for **1a–i** and **2a–i**

Compound	*C. albicans*	*C. tropicalis*	*C. kefyr*	*C. parapsilosis*	*C. krusei*	*C. dubliniensis*	ATCC 90028
1a	11.31	45.24	90.48	90.48	90.48	14.92	90.48
1b	43.10	43.10	86.20	43.10	86.20	43.10	86.20
1c	10.05	40.22	80.45	40.22	80.45	40.22	80.45
1d	21.23	42.47	84.95	42.47	84.95	42.47	84.95
1e	35.19	35.19	70.38	35.19	70.38	70.38	70.38
1f	45.24	45.24	90.48	45.24	172.22	45.24	45.24
1g	41.07	41.07	82.14	82.14	82.14	41.07	82.14
1h	0.54	3.42	27.38	109.53	13.41	13.69	54.76
1i	2.09	3.27	52.46	13.11	12.85	3.27	13.11
2a	45.24	11.31	5.65	22.62	2.82	5.65	90.48
2b	10.77	43.10	5.39	10.77	5.39	2.69	10.77
2c	40.22	20.11	5.02	10.05	40.22	40.22	80.45
2d	42.47	42.47	42.47	42.47	10.61	42.47	84.95
2e	35.19	8.79	4.39	8.79	4.39	2.19	70.38
2f	45.24	11.31	11.31	45.24	22.62	5.65	45.24
2g	82.14	41.07	41.07	10.26	5.13	41.07	82.14
2h	54.76	54.76	109.53	27.38	109.53	6.84	109.53
2i	6.55	3.27	26.23	13.11	13.11	6.55	13.11
Itraconazole	4.42	0.17	0.08	0.02	0.70	0.02	0.17
Fluconazole	1.63	313.44	1.63	1.63	104.48	1.63	1.63

naphthoquinones exhibit greater antifungal activity than β-compounds. There was marked variability among the different strains, with respect to their susceptibility to the test compounds, indicating that there are biological factor(s) affecting strain/species and drug bioactivity. For *C. albicans*, compound **1h** appeared to display the broadest antifungal activity, exhibiting a MIC value of 0.54 μM, as compared with 4.42 μM for Itraconazole and 1.63 μM Fluconazole. Molecule **1i** showed another interesting MIC value, 2.09 μM for *C. albicans*. Only compounds **1h** (MIC = 3.42 μM), **1i** (MIC = 3.27 μM) and **2i** (MIC = 3.27 μM) demonstrated promising antifungal activity for *C. tropicalis* in comparison with antifungal drug fluconazole (313.44 μM). Analysis of the results for strains *C. kefyr* and *C. krusei* revealed that substituted β-dihydrofuran naphthoquinones showed high antifungal activity, highlighting compounds **2a**, **2b**, **2c** and **2e**. For *C. dubliniensis*, compounds **1i** (MIC = 3.27 μM), **2b** (MIC = 2.69 μM) and **2e** (MIC = 2.19 μM) displayed low antifungal activity as compared with the standard drugs used. Strain *C. parapsilosis* and ATCC 90028 appeared to be the least sensitive to almost all of the compounds; only **2e** (MIC = 8.69 μM) demonstrated minimal antifungal activity for *C. parapsilosis*.

Additional toxicity tests, hemolytic activity in the blood of mice and cytotoxicity against NIH3T3 murine fibroblast culture using fluorescent Alamar Blue assay, were performed for all compounds (see ESI†). The studied compounds showed no significant hemolytic activity or cytotoxicity at concentrations of 50 μg mL^{-1} and 12.5 μg mL^{-1}. These results indicated that the tested compounds do not disrupt the cellular membrane or present unspecific cytotoxicity.

In conclusion, we have synthesized a series of substituted α- and β-dihydrofuran naphthoquinones, **1a–i** and **2a–i**, and evaluated them as antifungal agents against six strains of *Candida*.

The results indicated that **1h** was more active than commercially available drugs, itraconazole and fluconazole against *C. albicans*; **1i** also demonstrated good antifungal activity. Compounds **1h**, **1i** and **2i** exhibited a promising antifungal activity against strains *C. tropicalis*. *C. kefyr* and *C. krusei* were sensitive to compounds **2a**, **2b**, **2c** and **2e**. When compared with the standard drugs used, **1i**, **2b**, and **2e** showed discrete antifungal activity against *C. dubliniensis*. Strains *C. parapsilosis* and ATCC 90028 were the least sensitive to the naphthoquinones. Only compound **2e** demonstrated some antifungal activity against *C. parapsilosis*. Overall, some of the α-furan naphthoquinones exhibited potent antifungal activity, with no hemolytic activity or cytotoxic effects. These compounds are promising agents since only a few naphthoquinones are described in the literature as having antifungal activity.

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