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RESEARCH ARTICLE

2-Acryloyl-4,5-methylenedioxyphenol: A Small Molecule Endowed with Antidermatophytic Activity

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Abstract: ***Background*:** Superficial fungal infections are the most common fungal diseases in humans, affecting more than 25% of the population worldwide.Methods: In the present study, we have investigated the activity of kakuol, a natural compound isolated from the rhizomes of Asarum sieboldii, and some analogues, against various dermatophytes and pharmacologically relevant yeasts.

***Results*:** One of the tested compounds, 2-acryloyl-4,5-methylenedioxyphenol, showed a broad-spectrum activity against most of the fungal species assayed, resulting particularly effective against dermatophyte strains (MIC values in the range of 0.25-0.5 μg/mL, two/four-fold lower than the positive control miconazole).

***Conclusion*:** The results suggest that this molecule can be considered a promising starting point for the development of new antifungal compounds.

Keywords: Kakuol, *Asarum sieboldii*, antifungal agents, *Candida albicans*, dermatophytes, 3,4-methylenedioxyphenol.

1. INTRODUCTION

Superficial fungal infections involving skin, nails and mucosal surfaces, are the most common fungal diseases in humans, affecting more than 25% of the general population worldwide. Such infections are often recalcitrant to therapy and reduce the quality of life of infected individuals [1, 2]. Cutaneous and subcutaneous mycoses are caused by dematophytes like *Epidermophyton*, *Microsporum*, and *Trichophyton*, while mucosal infections are mainly caused by opportunistic yeasts belonging to the *Candida* genus [3].

Moreover, fungal species belonging to genera *Candida*, *Aspergillus*, *Cryptococcus*, *Mucor*, *Pneumocystis* produce invasive fungal infections associated with unacceptably high mortality rates. Such infections are very common in immuno-suppressed individuals as a result of aggressive therapies (*e.g.* anticancer chemotherapy, long-term corticosteroids treatment, or organ transplant) or immunosuppressive infections such as HIV/AIDS [1, 2].

Although many antifungal compounds have been developed during the last twenty years, they are confined to relatively few chemical classes. In addition, the emergence of fungal strains resistant to the current drugs on the market has reduced the effectiveness of the treatments [4-6]. Thus, the identification of novel scaffolds endowed with antifungal activity is becoming extremely urgent.

Naturally occurring substances from fungi, bacteria and higher plants are important sources of molecules with antifungal properties [7]. As a part of a research program aimed at developing new antifungal agents, we focused our attention on kakuol (compound **1**) (Fig. **1**), a secondary metabolite isolated from the rhizomes of *Asarum sieboldii* [8]. In a previous paper, we reported the activity of kakuol and a series of analogues against several plant pathogens [9]. The most promising derivative was 2-acryloyl-4,5-methylenedioxyphenol (compund **2**) (Fig. **1**), which showed a remarkable *in vitro* antifungal activity.

In the present study, we have extended our investigation to test the efficacy of kakuol and analogues numbered as compounds **2**-**4** in Fig. **1** against various pharmacologically relevant dermatophytes and yeasts.

2. MATERIALS AND METHODS

2.1. Chemistry

All reagents and solvents were reagent-grade or were purified by standard methods before use. Solvents were

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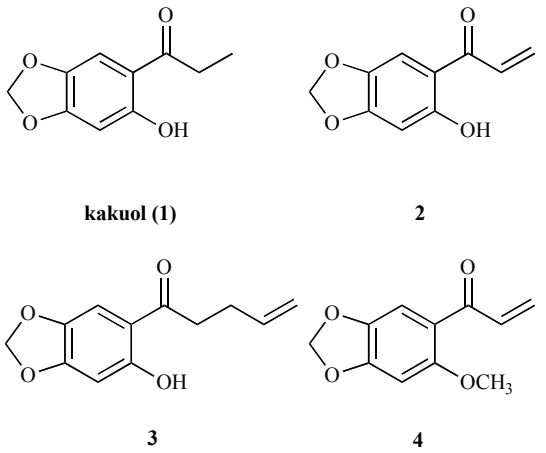


Fig. (1). Structure of the natural compound kakuol (**1**) and analogues **2**-**4**.

routinely distilled prior to use. Reactions requiring anhydrous conditions were performed under a positive nitrogen flow, and glassware was oven-dried. Anal. TLC: Fluka TLC plates (silica gel 60 F254, aluminium foil). Spectra: in $CDCl_3$ at r.t. on a Bruker AMX-300 spectrometer operating at 300 MHz; chemical shifts (δ) and coupling constants (*J*) are reported in ppm and in Hz, respectively. Kakuol and compounds **2** and **3** were prepared as previously reported [9].

1-(6-Methoxybenzo[1,3]dioxol-5-yl)propenone (Compound 4)

To a solution of compound **2** (50.0 mg, 0.26 mmol) in acetone (2 mL), K_2CO_3 (36.0 mg, 0.26 mmol) and CH_3I (88.0 mg, 0.62 mmol) were added and the resulting mixture was heated at reflux for 10h. The solvent was evaporated, then EtOAc (5 mL) and water (5 mL) were added. The organic phase was separated and dried with Na_2SO_4. Filtration and evaporation of the solvent under reduced pressure gave 59 mg (100%) of the title compound as a white sticky solid. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 7.30 (1H, s); 7.14 (1H, dd, *J* = 16.6, 10.4 Hz); 6.58 (1H, s); 6.32 (1H, dd, *J* = 16.6, 1.5 Hz); 6.02 (2H, s); 5.20 (1H, dd, *J* = 10.4, 1.5 Hz); 3.82 (3H, s). Anal. Calcd. for $C_{11}H_{10}O_4$: C. 64.07; H. 4.89. Found: 64.31; H. 4.87.

2.2. Biological Studies

2.2.1. Fungal Strains

Stocks of tested strains belonging to yeast (14 strains), filamentous (8 strains) and dermatophyte (14 strains) fungi, were either obtained from international institutes typified collections (American Type Culture Collection ATCC, National Collection of Pathogenic Fungi member of UKNCC, Istituto Superiore di Sanità, Rome), or were isolated in the hospital Istituto Dermopatico dell'Immacolata, Rome.

2.2.2. Antifungal Susceptibility Testing

Standard antifungal susceptibility testing was performed in accord with the Clinical and Laboratory Standards Institute's and European Committee on Antimicrobial Susceptibility Testing rules [10, 11] using RPMI 1640 medium (Glucose 2%) with L-glutamine, without sodium bicarbonate as sensitivity test medium, and Sabouraud broth as a growth medium to prepare the inocula.

2.2.3. Preparation of Inocula

Yeasts. An overnight culture of the fungi in Sabouraud broth was diluted 1:10 in the same sterile medium. The obtained fungal suspensions were adjusted with the use of 0.5 McFarland turbidity standard at $1x10^5$ cells/mL using the test medium (RPMI 1640).

2.2.4. Filamentous Fungi and Dermatophytes

Three-seven days old cultures in Sabouraud agar were harvested with a scraping device using Sabouraud broth supplemented with Tween 80 (0.5%). The obtained suspension of the conidia was counted at a Bürker camera and then standardized in the test medium (RPMI 1640) to $1x10^5$ cell/mL.

2.2.5. Preparation of Formulates

Test items (10 mg) were dissolved in DMSO (10 mL) and then diluted in the test medium (RPMI 1640) to reach the final value of 256 µg/mL. From this concentration, a series of dilutions down to 0.5 µg/mL, was performed.

2.2.6. Preparation of the Microtiter Plates

For each test item, 0.1 mL of previously prepared concentration, were dispensed (two for each concentration) into the wells of a microtiter. Then 0.1 mL of the adjusted fungal suspensions were added to each well containing the test item solutions. With this operation, a reciprocal dilution of 1:2 either for fungi or for test items, was obtained. The final concentrations were the following: yeast and filamentous fungi: 5×10^4 cells/mL; dermatophytes: 1×10^5 cells/mL; test items: from 128 µg/mL to 0.25 µg/mL in a 0.2 mL of final volume.

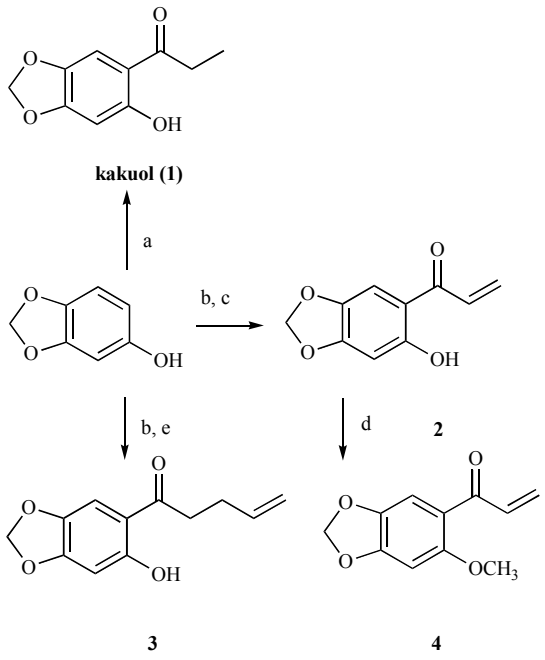
The microtiter plates were incubated 48 h at 35 °C for yeast and filamentous fungi, or 7 days at 28 °C for dermatophytes. The minimum inhibitory concentration (MIC) endpoint was defined as the lowest concentration that causes no visible turbidity or, at least, inhibition of growth ≥ 50% (yeasts) compared with the growth for a drug-free control.

2.2.7. In Vivo Experiments

Tolerability studies were performed using female athymic Swiss nude mice. Mice were maintained in laminar flow rooms keeping temperature and humidity constant. Mice had free access to food and water. Experiments were approved by the Ethics Committee for Animal Experimentation of the Istituto Nazionale Tumori of Milan according to institutional guidelines.

Compound **2** was suspended in ethanol-cremophor-water (10+10+80%) to a concentration of 10 mg/mL. Mice (8 weeks old) were administered orally by a single dose (200 mg/kg). Mice were weighed and monitored daily for 8 days after administration.

The results were as follows: body weight loss: (7%), lethal toxicity: 0/4. The necropsy of sacrificed animals on day 51 after treatment did not show any signs of disease.



Scheme 1. Synthesis of compounds 1-4. Reagents and conditions: **a**) $BF_3 \cdot Et_2O$, propanoic anhydride, 45 min, 80 °C; 73%; **b**) MeMgBr, Et_2O, 45 min, r.t.; **c**) CH_2=CHCOCl, toluene, r.t., 16 h, 66%; **d**) CH_3I, K_2CO_3, acetone, reflux, 10h, quantitative; **e**) CH_2=$CHCH_2CH_2COCl$, toluene, r.t., 16 h, 19%.

2.2.8. [1]H-NMR Study: Thiol-trapping Experiments

^{1}H NMR spectra were recorded at 25°C on a Bruker AV 600 spectrometer operating at a frequency of 600.13 MHz for 1H nucleus. All chemical shifts were reported in ppm (δ) and referenced to the chemical shifts of residual solvent resonances.

^{1}H NMR spectroscopy was used to determine the formation of the Michael adduct and allowed us to assess that the reaction was complete. No side products were detected.

The spectrum of compound **2** (25 mg) was acquired in DMSO-d_6 and all the signals were assigned. Cysteamine (20 mg), at molar ratio R = [cysteamine]/[**2**] = 2.0, was directly added into the NMR tube and the spectra were recorded at different times ranging from 5 to 60 minutes. The *in situ* generated adduct (30 microliters) was then diluted with $CDCl_3$ (1:20) in order to investigate the reversibility of the Michael reaction.

3. RESULTS AND DISCUSSION

3.1. Synthesis of Compounds 1-4

Kakuol (**1**) was obtained in a single step by reaction of sesamol with $BF_3 \cdot Et_2O$ in propanoic anhydride [9]. Compounds **2** and **3** were obtained by reacting the highly coordinating magnesium 3,4-(methylenedioxy)phenolate with the suitable acyl chlorides to introduce the desired acyl moiety directly at the *ortho* position of phenol group.

Compound **4** was prepared by treatment of compound **2** with CH_3I in acetone (Scheme **1**).

3.2. Antifungal Activity of Compounds 1-4

The antifungal activity of kakuol (**1**) against dermatophyte fungi and yeasts is presented in Tables **1**-**3**. In

Table 1. Minimum inhibitory concentration of compounds 1-4 and miconazole (MCZ) on dermatophyte fungi.

	MIC (μg/mL)				
Dermatophyte Fungi	**1**	**2**	**3**	**4**	MCZ
Trichophyton rubrum IDI D 1155	>32	0.5	64.0	2.0	2.0
Trichophyton mentagrophytes IDI D 1049	>32	0.5	64.0	2.0	2.0
Trichophyton quinckeanum NCPF 309	>32	0.5	64.0	2.0	2.0
Trichophyton rubrum SG 10 III		≤0.25			1.0
Trichophyton rubrum SG 9 II		≤0.25			0.5
Trichophyton mentagrophytes SG 1 I		≤0.25			0.5
Trichophyton soudanense SG 10 I		0.5			0.5
Trichophyton album SG IDI D 0250		8.0			8.0
Trichophyton violaceum IDI D 0086I		≤0.25			1.0
Microsporum canis IMM 3864	>32	0.5	≥64.0	64.0	2.0
Microsporum canis IDI D 1011		≤0.25			1.0
Microsporum gipseum SG 4 I		≤0.25			2.0
Epidermophyton floccosum SG 3 III	>32	0.5	≥64.0	16.0	2.0
Epidermophyton floccosum IDI D 0011		≤0.25			≤0.25

Table 2. Minimum inhibitory concentration of compounds 1-4 and miconazole (MCZ) on filamentous fungi.

	MIC (μg/mL)				
Filamentous Fungi	**1**	**2**	**3**	**4**	**MCZ**
Fusarium sp. F77	>64	2.0	≥64.0	8.0	16.0
Mucor mucedo ATCC 7941	>64	2.0	≥64.0	8.0	16.0
Penicillium sp. 1302	>64	8.0	≥64.0	64.0	4.0
Aspergillus niger ATCC 16404	>64	8.0	≥64.0	32.0	8.0
Aspergillus fumigatus ATCC 28212	>64	8.0	≥64.0	64.0	4.0
Aspergillus fumigatus G.S		16.0			4.0
Scopulariopsis brevicaulis SG 3 II		0.5			8.0
Acremonium sp. SG 11 III		4.0			8.0

Table 3. Minimum inhibitory concentration of compounds 1-4 and miconazole (MCZ) on yeasts.

	MIC (μg/mL)				
Yeasts	**1**	**2**	**3**	**4**	**MCZ**
Candida albicans ISS1	128.0	0.5	≥64.0	16.0	1.0
Candida albicans 562	128.0	8.0	≥64.0	8.0	8.0
Candida tropicalis ISS1	128.0	4.0	≥64.0	32.0	4.0
Candida krusei ISS1	128.0	0.5	≥64.0	8.0	1.0
Candida albicans IDI D 01011		4.0			16.0
Candida albicans 3575		4.0			16.0
Candida albicans SG 2 III		4.0			4.0
Candida albicans 700-94		4.0			16.0
Candida albicans PG		4.0			8.0
Candida albicans CA2		8.0			16.0
Candida guillermondii ISS 1		8.0			1.0
Candida krusei IDI D 1046		1.0			4.0
Candida tropicalis 5705		8.0			8.0
Saccharomyces cerevisiae ATCC7752	128.0	<0.25	≥64.0	2.0	<0.25

spite of the acceptable results obtained on pathogenic fungi of agricultural interest [8], kakuol showed only weak activity against all tested strains. We thus tested compound **2**, the most promising analogue from our previous investigation [9].

Interestingly, this molecule showed a broad-spectrum activity against most of the fungal species tested. It resulted particularly effective against dermatophyte strains (Table **1**), showing MIC values in the range of 0.25-0.5 μg/mL, two/four-fold lower than those of the positive control miconazole (MCZ). The only exception is represented by *Trichophyton album*.

Compound **2** was generally less effective on filamentous fungi, the MIC values being in the range of 0.5-16 μg/mL (Table **2**). Noteworthy, the MIC values of MCZ on these strains ranged between 4 and 16 μg/mL.

Moreover, the activity of compound **2** on yeasts was in the range of 0.5-8 μg/mL, comparable or lower than the activity of MCZ, with the exception of the *Candida guillermondii* ISS 1 strain (Table **3**).

To gain insights into the key features affecting the activity, analogues **3** and **4** were synthesized. The methoxy derivative **4** was prepared to investigate the role of the

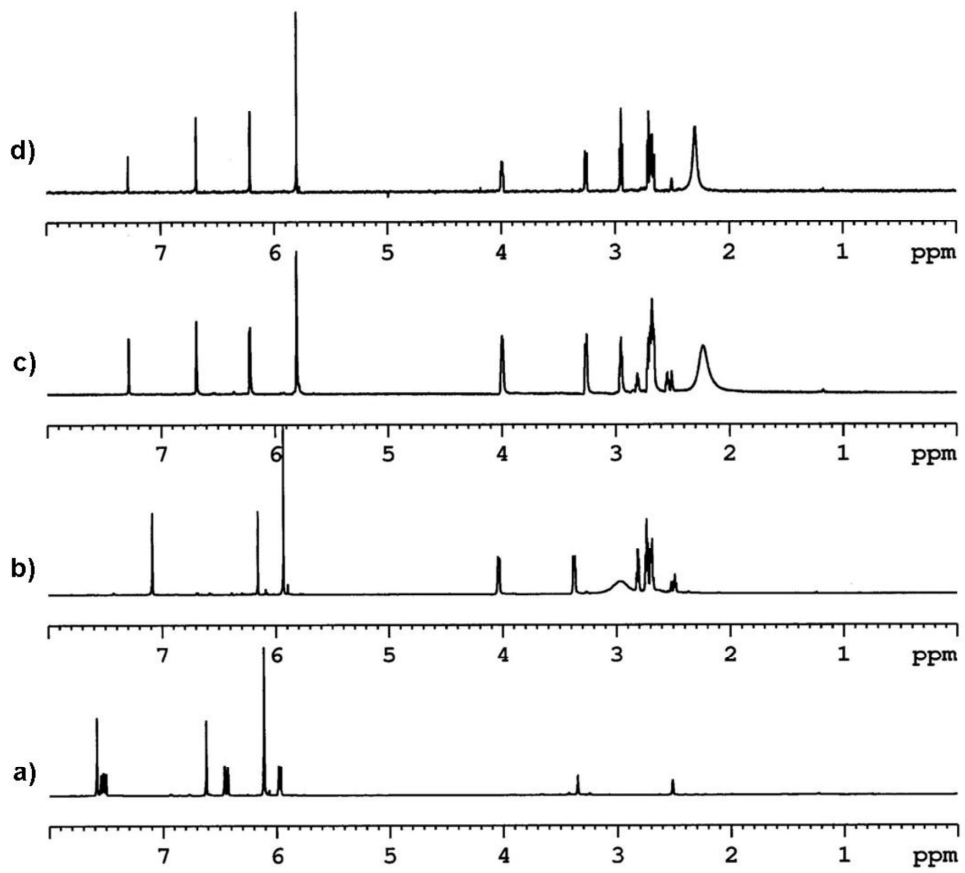


Fig. (2). (**a**) ^{1}H NMR spectrum of **2** in DMSO-d_6; (**b**) spectrum acquired 50 minutes after the addition of cysteamine at R = [cysteamine]/[**2**] = 2.0; (**c**) spectrum of the adduct acquired 5 minutes after the dilution with $CDCl_3$ (1:20); (**d**) spectrum of the adduct acquired 48 h after the dilution.

phenol group. MIC values of compound **4** against all the tested strains were generally higher than MICs of compound **2**, however lower than those of the natural compound kakuol (Tables **1**-**3**).

The role of the α,β-unsaturated carbonyl group was confirmed by examining compound **3** bearing a double bond two carbon atoms away from the carbonyl group. The compound resulted less effective than compound **2** against all the tested strains (Tables **1**-**3**). Overall, these results indicate that the conjugation between the double bond and the carbonyl group is a stringent structural requirement for the activity.

The α,β-unsaturated ketone functionality is well established as a Michael acceptor for trapping thiols [12]. Covalent binding of thiols represents an important mechanism of bioactivity and many biologically relevant pathways are targeted by thiol-reactive compounds [12]. Research on Michael acceptors has been recently rekindled by the FDA approval of the covalent kinase inhibitors afatinib and ibrutinib, containing acrylic acid amide groups [13].

To highlight the affinity of compound **2** towards thiols, we applied a simple NMR method recently developed by Avonto and coworker [14]. The ^{1}H NMR spectrum of compound **2** was acquired in DMSO-d_6 (Fig. **2**, see experiment for details) in the presence of cysteamine. After 50 minutes the total disappearance of signals attributed to the double bond and the appearance of the CH_2 signals proved that the addition of cysteamine to the double bond was quantitative. The solution of the in situ generated adduct was then diluted with $CDCl_3$: 48 hours after the dilution no signals belonging to the double bond reappeared (Fig. **2**), indicating that the addition of cysteamine to compound **2** did not show any solvent-induced reversibility.

The above observations suggest that, as expected, the reactive acrylic group of compound **2** is prone to a fast covalent binding mode to thiols. This can obviously cause concern with respect to its chemoselectivity [15].

However, it is noteworthy that in biological systems the reactivity of individual fragments strongly depends on the structure of the reacting proteins [16]. New approaches to identify preferred binding sites of covalently binding fragments have recently been developed [17]. Interestingly, reactive groups like acrylates in proteins have been found to give remarkable and rather unexpected chemoselectivity [18, 19]. Numerous research groups are currently involved in developing specific covalently binding inhibitors [16] and many approaches based on the use of covalent fragments as a starting point for lead-generation campaigns have been published to date [20, 21].

Preliminary *in vivo* studies, performed to shed light on the tolerability of compound **2**, showed that this molecule was not toxic when orally administered to mice in a single dose of 200 mg/kg (see experimental for details).

CONCLUSION

The present paper describes the evaluation of novel derivatives of the natural compound kakuol as potential antifungals for human treatment. In particular compound **2**, due to its remarkable *in vitro* activity, can be considered an interesting scaffold for the design of new chemotherapeutic agents. The results call for follow-up studies to further investigate the compound metabolism and its behaviour in biological systems.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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