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A Facile and Efficient Four-Step Enantioselective Synthesis of (+)-Vernolepin from (+)-Minimolide, the Major Germacranolide of *Mikania minima*

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Enantiomerically pure (+)-vernolepin was semi-synthesized for the first time using the synthon (6*S*,7*R*,8*S*)-8,14-diacetoxy-15-hydroxygermacra-1(10),4,11(13)-trien-6, 12-olide [(+)-minimolide], the major sesquiterpene lactone of the Argentinean vine *Mikania minima*. After performing four consecutive reactions (Cope rearrangement, two oxidations, and selective hydrolysis of the acetate groups) on the synthon (+)-minimolide, a (+)-vernolepin yield of *ca*. 40% was achieved, proving to be a suitable semi-synthetic strategy for the production of quantities between 0.5-1.0 g of (+)-vernolepin. The transformations described here mimetize the biogenetic pathway for the production of (+)-vernolepin in the genus *Vernonia*. The synthesized (+)-vernolepin, but not its precursors, shows antifungal activity similar to amphotericin B. The semi-synthesis reported here combines affordable and easily available chemical reagents with classical organic methodologies.

Keywords: (+)-vernolepin, (+)-minimolide, semi-synthesis, antifungal activity.

The sesquiterpene lactones (+)-vernolepin (**1**), (-)-vernomenin (**2**), and (+)-vernodalin (**3**) have been isolated from the African ethnomedicinal plants *Vernonia hymenolepis* and *V. amygdalina* (Compositae) [1-4]. These naturally occurring lactones, along with the synthetic derivative 8–deoxyvernolepin (**4**) [5], belong to a small elemane bis-lactones group characterized by the presence of a 10-vinyl-2-oxa-*cis*-decalin skeleton.

Previous studies have reported that these lactones show remarkable biological properties. Arguably, the most extensively studied compound from this sesquiterpene lactone group is **1**, which possesses a high tumor inhibition activity *in vitro* and *in vivo* [3,4,6], spasmolytic, anti-aggregating, de-aggregating [7], and antiparasitic [8] properties, plant growth inhibition [9], and a remarkable antibacterial activity shown to be even higher than ampicillin and neomycin [10]. Surprisingly, **1** also possesses a potent antifungal activity comparable to amphotericin B [10]. These properties, along with its unusual structure, have attracted the attention of several research groups since the '70s. Although several groups attempted to totally synthesize **1** or structurally related compounds, the number of steps involved along with the low yields obtained (mixtures of stereoisomers or racemic products) pointed to the complexity to obtain a total and efficient chemical synthesis of **1** [11].

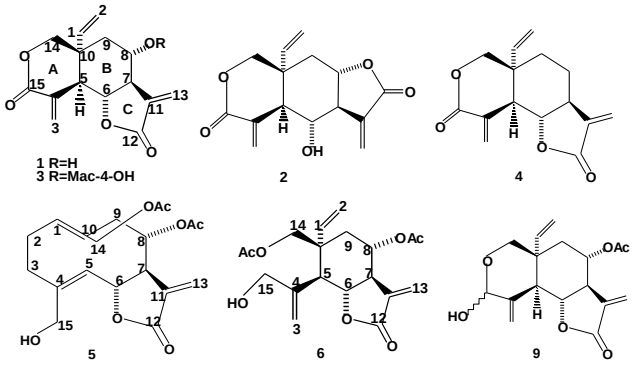


Figure 1: Chemical structures of minimolide, vernolepin and analogs.

Since 1994, several research groups started to consolidate the idea of replacing the laborious and inefficient methodology of total synthesis of **1** with a semi-synthetic strategy based on the use of natural synthons as starting materials [12-15]. Although natural synthons containing germacrolide skeletons have been used for the semi-synthesis of **1**, an efficient methodology has not been reported yet [16].

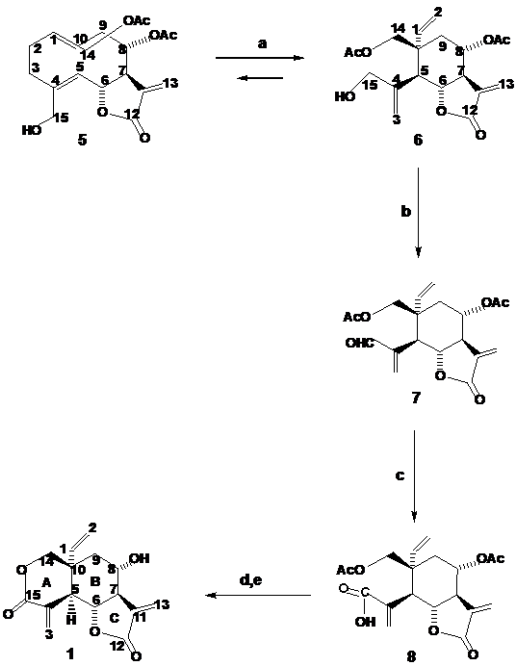
Previous studies from our group on *Mikania minima* (Bak.) Robinson [17] reported the presence of a crystalline germacranolide, (6*S*,7*R*,8*S*)-8,14-diacetoxy-15-hydroxy-germacra-1(10),4,11(13)-trien-6, 12-olide (**5**), for which we propose the name "minimolide", as the major sesquiterpene lactone (yield 0.8-1.1% w/w based on air-dried leaves) of this plant. The structure and absolute configuration of **5** was securely solved by X-ray diffraction analysis [18] and was then proposed as a synthon for the semi-synthesis of **1**, based also on the fact that the elemanolide-type isomers **6** and **7** co-occur with **5** in the same plant [17,18].

Therefore, our approach to consolidate the semi-synthesis of **1** was:
1) To optimize the isolation of crystalline **5** from *Mikania minima*.
2) To convert **5** into **6** (Scheme 1, a) following a Cope rearrangement.
3) To oxidize the primary allylic alcohol group at C-15 of **6** to the correspondent α,β-unsaturated carbonyl (**7**) (Scheme 1, b), and then to carboxyl group (**8**) (Scheme 1, c).
4) To hydrolyze the acetate group at C-8 and C-14 of **8** promoting the spontaneous lactonization between the primary hydroxyl at C-14 and the carboxyl at C-15 to complete the synthesis of **1** (Scheme 1, d and e).

To confirm that the product obtained possesses also the antifungal activities previously reported for **1** [10], the compounds **1**, **5**, **6**, **7**, and **8** were tested against human pathogenic fungal strains. A minimum inhibitory concentration (MIC) of 3 µM against *Aspergillus niger* and *Cryptococcus neoformans* was measured for **1,** while no antifungal activity was detected when *Candida albicans* and *Tricophyton rubrum* were assayed. The same MIC of **1** was also recorded when amphotericin B was evaluated as a positive control. No antifungal activity was recorded when the intermediates **5**, **6**, **7**, and **8** were assayed. We are not able to compare our results with a previous work reporting the antifungal activity of **1** [10] because of the lack of information about how the compound was prepared and how the assay was performed.

Recently, **3** (Mac-4-OH = 4-hydroxymethacrylate) and its 11,13-dihydroderivative have been reported as the major lactones in *Distephanus angulifolius* (syn. *Vernonia angulifolia*) [19]. **3** is an analog of **1** differing only in the presence of 4-hydroxy-methacrylate ester side chain at C-8. Interestingly, the same study reports the isolation of an elemanolide-type lactone analogue to **7,** differing also for having a Mac-4-OH chain at C-8. The simultaneous presence of germacranolides and elemanolides in *D. angulifolius* strongly supports a similar biogenetic pathway for the production of **1** (and analogues) in the genus *Vernonia*. Accordingly, the biogenesis of **1** and its relatives would consist of the following sequential steps: a germacra-1(10),4-dienolide functionalized at C-8 and C-14 → Cope rearrangement to the correspondent elemanolide → oxidation of the primary allylic alcohol group at C-15 to α,β-unsaturated aldehyde → oxidation of the α,β-unsaturated aldehyde to the correspondent α,β-unsaturated acid → hydrolysis of the ester group at C-14, followed by either spontaneous or enzyme catalyzed lactonization between the primary alcohol at C-14 and the C-15 carboxyl group to yield the δ-lactone ring characteristic of the elemane bis-lactones group. Alternatively, hydrolysis of the ester side chain at C-14 of the α,β-unsaturated aldehyde **7** followed by intramolecular hemiketalization would yield **9**, which can then be oxidized to **1**. Indeed, our first attempts to obtain **1** started with aldehyde **7**, which can easily be obtained in high yield by Jones oxidation of **6**. However, several attempts using different strategies to hydrolize the acetate groups of **7** produced an undesirable 1,4-addition of water (and other nucleophiles) to the α,β-unsaturated aldehyde system (Michael addition). Also, several treatments of **7** with pig liver esterase and porcine pancreatic lipase were unsuccessful, and only a partial γ-lactone ring opening was observed.

Taking together, we report here for the first time and to the best of our knowledge, the enantiospecific four-step semi-synthesis of **1** from a germacranolide synthon with a global yield of 40%. This protecting-group-free semi-synthesis, based on the use of affordable and commonly available reagents, and a simple methodology with an attractive overall yield, paves the way for the development of a synthetic method to be considered for industrial large-scale production.



Scheme 1: (a) Cope rearrangement in toluene, reflux, 5 h, in N_2 atmosphere, 68%; (b) Jones reagent: CrO_3, H_2SO_4, acetone, 0˚C, 30 min, 93%; (c) Sodium chlorite, NaH_2PO_4, *t*-BuOH/2-methyl-2-butene, 88%; (d) *p*-dioxane, HCl 1.5%, 60-65˚C; (e) Benzene, *p*-toluenesulfonic acid (cat.), reflux, 73%.

Experimental

General experimental procedures: NMR measurements were recorded on a Bruker 200 AVANCE. Merck silica gel (230-400 mesh, ASTM) was used for column chromatography (CC).

(6S,7R,8S)-8,14-Diacetoxy-15-hydroxygermacra-1(10),4, 11(13)-trien-6,12-olide [(+)-minimolide] (5): 1.0 kg of dried aerial parts (leaves and flower heads) of *Mikania minima* were extracted (2x) with 5 L of CH_2Cl_2 at room temperature during 10 min with occasional stirring. After filtering through cotton wool, most of the solvent was distilled off using a Vigreux column. From the concentrated extract, the residual solvent was evaporated using a rotary evaporator at reduced pressure, followed by 20 min in a vacuum desiccator. A total of 65 g of crude extract (6.5% yield) was obtained. The extract was then suspended in 560 mL of EtOH 96º at 50-55ºC and then diluted with 400 mL of H_2O. The mixture was extracted successively with hexane [300, 200, and 150 mL (3x)], and benzene [300, 200, and 150 mL (4x)]. The benzene extracts containing **5** were combined, dried with Na_2SO_4, filtered through a Whatman No. 1 filter paper, and the solvent was removed in a rotary evaporator to afford a gummy residue. This residue was immediately poured into a 1 L beaker and ethyl ether was added in portions (3 x 100 mL) and stirred continuously using a glass rod until an amorphous solid was observed. This process takes 4-5 h. The supernatant solution was decanted, the amorphous solid was dissolved using the minimum amount of fresh ethyl ether, and left to evaporate slowly at room temperature until one fourth of the initial volume. Crystalline needles of **5** were collected by decantation and recrystallyzed using a 7:3 mixture of EtOAc-heptane. Additional amounts of **5** can be recovered from the mother liquors. Yield: 8.5-11.2 g (0.85-1.12%).

NB: While crystalline **5** can remain stable for several years at -18˚C, in its amorphous state polymerizes after a few days or weeks. The previously unreported optical rotation of **5** is given below.
MP: 112ºC (from EtOAc-*n*-heptane) (reported [18]): 112-113ºC.
$[\alpha]_D$: +61.8 (*c* 4.66, $CHCl_3$).
^{1}H- and ^{13}C-NMR identical to those reported [17].
MS (ion trap, 70 eV, direct inlet): *m/z* (%) 365 $[M+H]^+$ (14), 364 (10), 347 (11), 346 (10), 305 (25), 287 (31), 245 (44), 227 (100), 199 (38), 181 (38), 171 (18), 169 (19), 91 (20).

(5S,6R,7R,8S,10S)-8,14-diacetoxy-15-hydroxy-elema-1,3,11(13)-trien-6,12-olid (6): 10 g of **5** dissolved in toluene (2 L) was refluxed under a N_2 atmosphere until the reaction reached the equilibrium (ca. 5 h), as determined by TLC and ^{1}H-NMR analysis. The reaction mixture was filtered through Whatman No. 1 filter paper and the solvent evaporated at reduced pressure. The residue was chromatographed on a Si gel column (230–400 Mesh) packed with *n*-hexane. The residue, suspended in a mixture of *n*-hexane/EtOAc 4:3, was placed at the top of the column and eluted isocratically with the same solvent mixture. TLC was used to monitor the progress of the chromatography. After elution of **6**, the polarity of the elution mixture was increased to accelerate the complete elution of **5**. The ^{1}H-NMR spectrum recorded for the Cope equilibrium mixture revealed that in the steady-state equilibrium, the composition of the mixture contained 68% of **6** and 32% of **5.** This percentage was calculated by integration of the signals corresponding to H-13a, H-13b and H-7 of both lactones. The yield of isolated **5** and **6** were 2.15 g (21.5%) and 5.61 g (56.1%) respectively. Recovered **5** can be recycled to produce an additional amount of **6**.
Gum
$[\alpha]_D$: +66.2 (*c* 7.1, $CHCl_3$).
^{1}H- and ^{13}C-NMR identical to those reported [16].
MS (ion trap, 70 eV, direct inlet): *m/z* (%) 365 $[M+H]^+$ (27), 347 (22), 305 $[M+H\text{-}AcOH]^+$ (23), 287 (26), 245 (28), 227 (100), 199 (26), 181 (38).

(5S,6R,7R,8S,10S)-8,14-diacetoxy-15-oxo-elema-1,3,11(13)-trien-6,12-olid (7): This aldehyde was prepared from **6** using four different oxidation techniques [20-22]. The following methods were used:

a). Oxidation with chromium trioxide (CrO_3): 83 mg (83 mmol) of chromium trioxide was dissolved in a mixture of 0.13 mL pyridine (1.68 mmol) and 2 mL of dry CH_2Cl_2. This suspension was mixed with 0.16 mL of dry CH_2Cl_2 containing 54 mg (0.15 mmol) of **6**. The mixture was magnetically stirred during 1.5 h at room temperature and the progress of the reaction was monitored by TLC. The oxidation was complete after 1.5 h, and a single spot corresponding to **7** was observed [20]. The reaction mixture was filtered through a short Si gel column (230-400 Mesh) using $CHCl_3$ as elution solvent. The chloroformic filtrate was washed successively with 1 mL HCl 10% (2x), brine (1.5 mL), and dried with anhydrous Na_2SO_4. After filtering and solvent evaporation, 49.8 mg of **7** was obtained (yield 92%).
Gum
$[\alpha]_D$: +39.2 (*c* 5.4, $CHCl_3$).
^{1}H- and ^{13}C-NMR identical to those reported [18].
MS (ion trap, 70 eV, direct inlet): *m/z* (%) 363 $[M+H]^+$ (68), 303 $[M+H\text{-}AcOH]^+$ (93), 243 (100), 214 (48), 213 (51), 197 (71), 185 (43), 169 (42), 91 (57).

b). Oxidation with pyridinium chlorochromate (PCC): This oxidation was carried out according to published protocols [21]. Briefly, a suspension of 709.5 mg PCC (3.3 mmol) dissolved in 4.4 mL of dry CH_2Cl_2 was magnetically stirred with 1010 mg (2.78 mmol) of an equilibrium mixture of Cope rearrangement (containing a 32:68 equilibrium ratio of **5**:**6**). TLC was used to monitor the progression of the reaction, which was completed after 90 min. Next, 10 mL of dry ethyl ether was added to this

reaction mixture and the ethereal supernatant was separated from the gummy dark residue by decantation. This residue was then washed with 3 mL of dry ethyl ether (3x), and the combined ethereal phases were chromatographed through a Florisil column to afford 646 mg of essentially pure **7** (yield: 94% based on the amount of **6** in the equilibrium mixture).

c). Oxidation with Jones reagent (chromic acid in acetone): This oxidation was carried out according to published protocols [22]. Briefly, 0.5 mL of this reagent was added dropwise to 364 mg (1 mmol) of **6**, previously dissolved in distilled acetone at 0°C under continuous stirring. TLC was used to monitor the progression of the reaction. After 20 min, the reaction was completed; the organic layer was decanted, the solvent evaporated, and the residue was chromatographed through a short column of Si gel (230-400 Mesh) using a mixture of $CHCl_3$:EtOAc (90:10) to yield 341 mg of **7** (gum, 93.7%).

d). Oxidation with active MnO_2 in $CHCl_3$: 450 mg of active MnO_2 was added to **6** (73 mg; 0.2 mmol) dissolved in $CHCl_3$ (50 mL) at room temperature with magnetic stirring. The progress of the reaction was monitored by TLC and completed after 2.5 h. Filtration and solvent evaporation yielded **7** (65 mg; 90%).

***(5S,6R,7R,8S,10S)-8,14-diacetoxy-elema-1,3,11(13)-trien-6,12-olide-3-oic acid (8)*:** This acid was prepared from aldehyde **7** using two different oxidation techniques.

a). Oxidation with Jones reagent: This oxidation was performed according to a published protocol [23]. Briefly, to 167 mg (0.46 mmol) of **7** dissolved in freshly distilled acetone (15 mL), a large excess of Jones reagent (7 mL) [22] was added dropwise at room temperature with continuous stirring. The progression of the reaction was followed by TLC and the reaction was completed after 29 h. 100 mg of $NaHSO_3$ dissolved in 10 mL water was used to eliminate the excess of Jones reagent. The reaction mixture was then extracted with 15 mL of $CHCl_3$ and the acid aqueous phase was extracted twice with 10 mL of $CHCl_3$. The chloroform extracts were combined, washed with brine (2x), and dried (Na_2SO_4). After filtering and solvent evaporation, 122 mg of crude acid **8** was recovered. This crude acid was chromatographed on Si gel column (230–400 Mesh) with $CHCl_3$:EtOAc (1:1) as eluent to yield 65 mg (37%) of pure **8** as a gum.

$C_{19}H_{22}O_8$
MW: 378.13 g/mol.
^{1}H NMR (300 MHz, $CDCl_3$): δ 1.65 (1H, dd, *J* = 13.5, 10.7 Hz, H-9a), 2.13 (3H, s, acetate methyl), 2.12 (3H, s, acetate methyl), 2.40 (1H, dd, *J* = 13.5, 4 Hz, H-9b), 2.92 (1H, tt, *J* = 11, 2.7 Hz, H-7), 3.26 (1H, d, *J* = 12 Hz, H-5), 4.07 (1H, d, *J* = 11.8 Hz, H-14b), 4.27 (1H, d, *J* = 11.8 Hz, H-14a), 4.61 (1H, dd, *J* = 12, 11 Hz, H-6), 5.02 (1H, d, *J* = 17.6 Hz, H-2b), 5.12 (1H, d, *J* = 11 Hz, H-2a), 5.27 (1H, dt, *J* = 4, 10.7, 10.7 Hz, H-8), 5.62 (1H, d, *J* = 2.7 Hz, H-13b), 5.67 (1H, dd, *J* = 17.6, 11 Hz, H-1), 5.76 (1H, s, H-3b), 6.18 (1H, d, *J* = 2.7 Hz, H-13a), 6.68 (1H, s, H-3a).
^{13}C NMR (75 MHz $CDCl_3$): 20.9 (acetate methyls x 2), 40.4 (C-9), 44.5 (C-10), 49.1 (C-5), 51.6 (C-7), 65.4 (C-14), 69.1 (C-8), 77.0 (C-6), 115.0 (C-2), 120.4 (C-13), 131.6 (C-4), 134.1 (C-3), 136.4 (C-11), 140.6 (C-1), 169.1 (C-12), 170.5 and 170.1 (acetate carbonyls), 171.1 (CH_2, C-15).
MS (CI, isobutane): *m/z* (%) 379 $[M+H]^+$ (21), 319 (32), 259 (18), 258 (21), 240 (27), 230 (30), 213 (59), 212 (92), 145 (54), 117 (100), 116 (96), 91 (97).

b). Oxidation with sodium chlorite: This reaction was performed according to a published protocol [24]. A solution of $NaClO_2$ (801 mg, 8,85 mmol) and $NaH_2PO_4.2H_2O$ (941 mg, 6.03 mmol) in H_2O (7.7 mL) was added dropwise into a mixture of aldehyde **7** (384 mg, 1.06 mmol) and 2-methyl-1-2-butene (4.5 mL) in *t*-BuOH (22.5 mL). The mixture was stirred for 4 h and the volatile organic compounds were removed *in vacuo*. The residue was diluted with H_2O (15 mL) and extracted with 25 mL of $CHCl_3$ (4x). The organic extracts were combined, dried over anhydrous Na_2SO_4, and filtered. After solvent evaporation, the residue was flash chromatographed through a short pad of Si gel (70-230 Mesh) using $CHCl_3$:EtOAc (1:1). After solvent volatilization, 352 mg (88%) of essentially pure **8** (TLC and NMR analysis) was obtained.

(+)-Vernolepin (1): Selective hydrolysis of the acetate residues at C-8 and C-14 of compound **8** was achieved by using HCl in aqueous dioxane. A solution of **8** (189 mg, 0.5 mmol) in freshly distilled dioxane (10 mL) was mixed with 10 mL of 3% HCl at room temperature. The solution was then heated at 60–65°C for 2.5 h in a thermic bath. TLC monitored the progression of the reaction. The reaction mixture was cooled and 20 mL of a saturated brine solution was added. The mixture was then placed in a decantation funnel and extracted with 30 mL of EtOAc (5x). The combined organic phases were dried with anhydrous Na_2SO_4, filtered through cotton, and the solvent evaporated at reduced pressure. The residue was dissolved in anhydrous benzene (100 mL), a catalytic amount of *p*-toluenesulfonic acid was added, and 50 mL of benzene was distilled off using a Dean-Stark trap. The remaining solvent was removed *in vacuo* and the residue was chromatographed on Si gel (230–400 Mesh) using a mixture of $CHCl_3$:EtOAc to yield 7 mg (5%) of **2**, and 101 mg (73%) of **1**. Both **1** and **2** were identified by their spectroscopic data [25].

$C_{15}H_{16}0_5$
MW = 276.10 g/mol.
MP: 178-180°C (from EtOAc-*n*-heptane) reported [3]: 179-180°C.
$[\alpha]_D$:+70.5 (*c* 3.4, acetone) (reported [4]: $[\alpha]_D$: +72).

^{1}H NMR (200 MHz, $CDCl_3$): 1.67 (1H, dd, *J* = 14.2, 10.3 Hz, H-9b), 1.99 (1H,dd, *J* = 14.2, 4.6 Hz, H-9a), 2.07 (1H, br s, OH), 2.69 (1H,dddd, *J* = 11, 10.5, 3.3 Hz, H-7), 2.98 (1H, dd, *J* = 11.7, 1.6 Hz, H-5), 3.96 (1H, dd, *J* = 11.7, 11 Hz, H-6), 4.09 (1H, ddd, *J* = 10.5, 10.3, 4.6 Hz, H-8), 4.23 (1H, dd, *J* = 12.0, 1.6* Hz, H-14b), 4.42 (1H, d, 12.0 Hz, H-14a), 5.23 (1H, d, *J* = 17.5 Hz, H-2b), 5.27 (1H, d, *J* = 17 Hz, H-2a), 5.73 (1H, dd, *J* = 17.2, 11.1 Hz, H-1), 6.04 (1H, d, *J* = 3.0 Hz, H-13b), 5.95 (1H, br s, H-3b), 6.24 (1H, d, *J* = 3.0 Hz, H-13a), 6.74 (1H, br s, H-3a).* W-type coupling with H-5 confirms the *cis* fusion of the δ-lactone ring.

MS (EI, 70 eV, GC-MS): *m/z* (%) 276 $[M]^+$ (2), 258 (3), 246 (5), 228 (25), 213 (7), 200 (13), 182 (28), 122 (64), 121 (93), 107 (53), 97 (65), 91 (100), 79 (54), 77 (66), 69 (77), 53 (37), 43 (43), 41 (79).

Antifungal activity: The antifungal activity of **1** and its intermediates in the semi-synthesis of **1** was assayed against *Aspergillus niger* (ATCC 32656), *Trichophyton rubrum* (ATCC 18758) *Candida albicans* (ATCC 14053), and *Cryptococcus neoformans* var. H99 (provided by Dr. J. Kronstad, Michael Smith Laboratories, University of British Columbia). Compounds **1**, **6**, **7**, and **8** were dissolved in DMSO to a final concentration of 100 μM, and serial dilutions of 50, 10, 5, 4, 2.5, 1.25, 0.125 μM were prepared. Tests were performed using 96-well ELISA microplates. Each well contained 3 μL of each compound**,** 97 μL of Sabouraud broth and 2 or 10 μL of a spore suspension of *A. niger* or *T. rubrum*, respectively. In the case of yeasts, 5 μL of an overnight culture of either *C. albicans* or *C. neoformans* at an O.D. of 0.1 (at 625 nm) was added. The final concentration of DMSO in each well did not exceed 3%. Fresh Sabouraud broth, 3% DMSO, and pure cultures (without treatment) were used as negative controls, while the antifungal amphotericin B was used as a positive control at the same concentrations tested for the compounds. All the assays were performed in triplicate.

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