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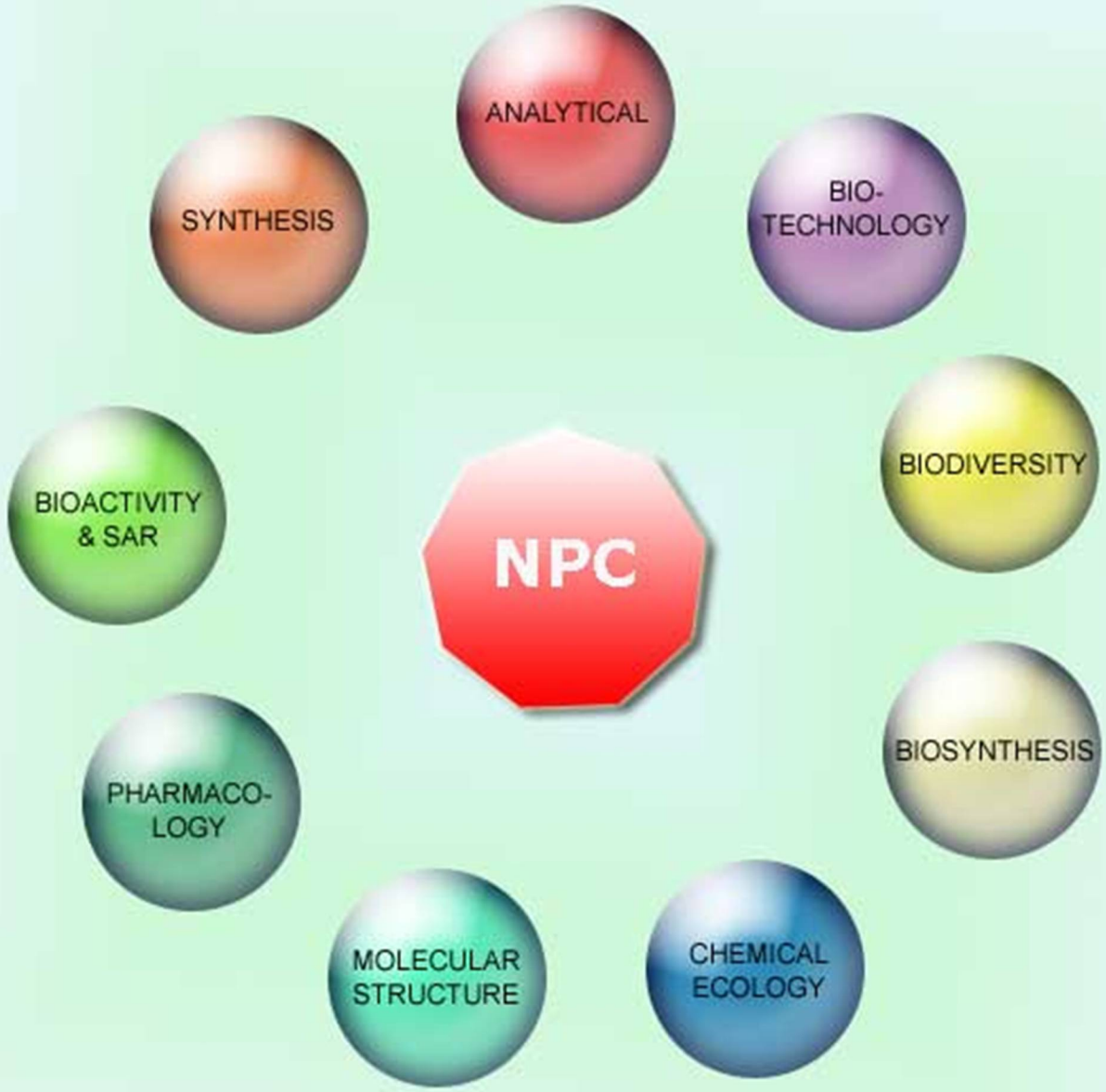

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Synthesis and Biological Evaluation of Combretastatin A-4 and Three Combretastatin-Based Hybrids

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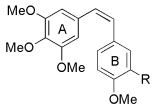
The syntheses of combretastatin A-4 from gallic acid and of three combretastatin-based hybrids are described. Starting from commercial gallic acid, the phosphonium salt (3,4,5-trimethoxybenzylphosphonium bromide) is synthesized and coupled, through a Wittig reaction, with several aldehydes, including methoxymethyl-protected isovanillin, the aldehyde γ-bicyclohomofarnesal having a labdane skeleton, 3-(3-pyridyl) propanal, and furfural. The biological properties of the *cis*-coupled compounds as cytotoxic, antiviral and antifungal agents are also reported. In addition, pyrogallol, gallic and 3,4,5-trimethoxybenzoic acids have been studied biologically.

Keywords: Combretastatin A-4, Gallic acid, Pyrogallol, Antifungal activity, Cytotoxic activity, Antiviral activity.

Combretastatin A-4 (CA-4, **1**) (Figure 1) is isolated from the African willow tree *Combretum caffrum* and has been known for over 20 years ago [1]. It is a strong tubulin depolymerizing agent and, therefore, inhibits tumor growth and has antivascular effects [2]. Its prodrug (disodium salt, water-soluble phosphate derivative, CA-4P, **2**) has entered clinical trials for both solid and liquid tumors [3]. Similarly, a close derivative of CA-4 (AVE-8062, **3**) is also undergoing clinical trials [4]. Both compounds have demonstrated a sufficient safety profile in phase I trials, suggesting that this may be a viable therapeutic strategy.



1, Combretastatin A-4 (CA-4) R=OH
2, Combretastatin A-4P (CA-4P) R=OPO_3Na_2
3, AVE-8062 R=NH-Serine

Figure 1: Tubulin polymerization inhibitors.

Despite its low molecular weight and simple molecular structure, CA-4 is one of the most powerful inhibitors of tubulin polymerization known to date. The structure can be divided into three separate components, the two rings (usually termed A and B) and the olefinic bridge. The structure-activity relationship (SAR) of CA-4 has been investigated thoroughly by a number of groups, being reasonably well-understood, and medicinal chemistry has been largely explored on its carbon framework [5]. In brief, it is thought that the *cis*-configuration of the olefinic bridge, the presence of the 3,4,5-trimethoxy group on ring A, and the *para*-methoxy group on ring B are fundamental for antitubulin activity. However, active compounds not obeying this general rule have been described [6]. Thus, modifications of the CA-4 structure have led to a great number of novel CA-4 derivatives as potent tubulin inhibitors. Among these derivatives, a number of combretastatin-based hybrids have been the objective of several research groups, for example, the synthesis and biological properties of combretastatin-based hybrids with colchicines [7], lamellarins [8], chromone [9], and chalcone [10]. On the basis of this motivation, in this study, and based on our prior experience with other bioactive

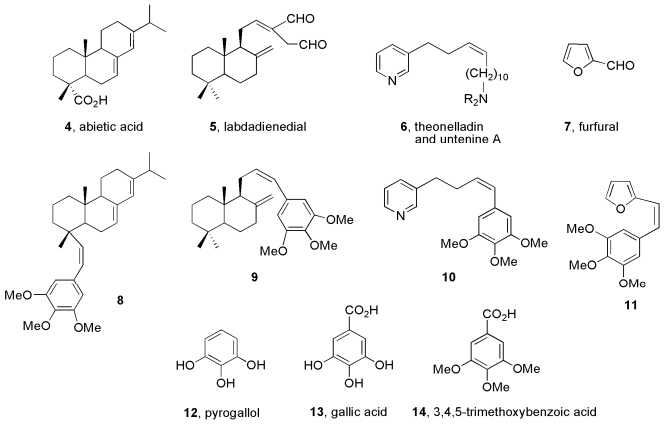


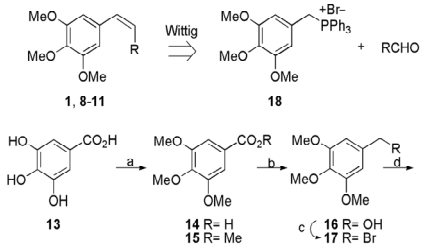
Figure 2: Designed combretastatin-based hybrids, parent carbon frameworks and other tested molecules.

molecules, we planned to select the 3,4,5-trimethoxybenzene and the *cis*-olefinic bridge moieties present in CA-4 as the core structure. Therefore, in this study we have combined important pharmacophore structures of combretastatins with bioactive carbon frameworks to design several hybrids, which have been studied as cytotoxic, antiviral and antifungal agents (Figure 2). In addition, pyrogallol (**12**), gallic acid (**13**) and 3,4,5-trimethoxybenzoic acid (**14**) (trimethylgallic acid) have also been studied biologically.

As parent carbon frameworks to combine with the 3,4,5-trimethoxybenzene moiety through a *cis*-olefinic bridge, we have chosen two terpenoid skeletons that have shown a high bioactive profile, abietic acid (**4**) [11], and the natural dialdehyde belonging to the labdane class of terpenoids, labdadienedial (**5**) (Figure 2) [12]. Also, we wanted to introduce a 3-alkylpyridine moiety, which is present in natural bioactive pyridine alkaloids **6**, as antineoplastic theonelladin A and anti-microfouling untenine A [13]. Finally, we wanted to introduce a furan ring, through furfural **7**, to see the influence of this on bioactivity.

Our retrosynthesis towards CA-4 and the combretastatin-based hybrids focused on the Wittig reaction (Scheme 1) between the known phosphonium salt, 3,4,5-trimethoxybenzylphosphonium bromide (**18**) [14], which was synthesized from gallic acid (**13**), and several readily available aldehydes, such as methoxymethyl-protected isovainillin **20** (Scheme 2) [15], and the aldehyde abietinal (**23**), prepared from abietic acid [11], the aldehyde γ-bicyclohomofarnesal (**26**) prepared from (+)-sclareolide [16], 3-(3-pyridyl)propanal (**28**) prepared by Dess-Martin oxidation of 3-(3-pyridyl) propanol (**27**) [13b], and commercial furfural **7**. Aldehydes **23** and **26** are known intermediates in the synthesis of different terpenoid systems and both can be prepared in three steps starting from commercial (-)-abietic acid and commercial (+)-sclareolide, respectively [11,12]. The key Wittig reaction with ylide derived from phosphonium salt **18** has precedent in the literature, and has already been reported with different protecting groups in the aldehyde counterpart in the synthesis of combretastatin A-4 [17]. Thus, the introduction of the 3,4,5-trimethoxybenzene and *cis*-olefin moieties to obtain CA-4 was easy, but, the introduction of these moieties into other aldehyde systems represented a challenge for us.

The synthesis began with the alkylation and esterification of gallic acid (**13**) using dimethyl sulfate in basic media to afford trimethoxy-ester (methyl 3,4,5-trimethoxybenzoate) **15** (80%) (Scheme 1).

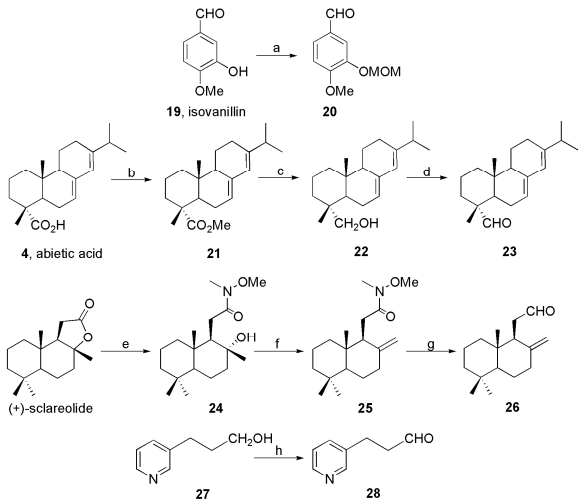


Reagents: a)Me_2SO_4,NaOH; b)$LiAlH_4$, THF; c)CBr_4,PPh_3; d) PPh_3,PhMe

Scheme 1: Retrosynthesis of the combretastatin-based hybrids and synthesis of the phosphonium salt 18 from gallic acid [13].

The control of the esterification step led to the isolation of trimethylgallic acid (92%, **14**) as intermediate. Then, reduction of the ester in **15** with $LiAlH_4$ in THF gave the corresponding benzyl alcohol **16** in 88% yield, which was converted to its corresponding bromide **17** (80%) by reaction with CBr_4/PPh_3 in DCM. Finally, treatment of bromide **17** with triphenylphosphine in refluxing toluene led to the key phosphonium salt **18** in quantitative yield (Scheme 1) [14].

Next, we carried out the synthesis of the necessary aldehydes for the Wittig reaction with the ylide derived from phosphonium salt **18** (Scheme 2). First, we protected commercial isovanillin (**19**) as its corresponding methoxymethyl ether using $MOMCl/K_2CO_3$ to afford MOM-ether **20** in 76% yield [15]. Abietic acid (**4**) was esterified by treatment with lithium hydroxide and dimethyl sulfate to give ester **21** in quantitative yield. Reduction of ester **21** with $LiAlH_4$ in dry tetrahydrofuran under reflux gave alcohol (abietinol) **22** in quantitative yield. Oxidation of **22** was carried out by treatment with PCC on alumina [18]. This oxidation afforded the desired aldehyde (abietinal) **23** in 40% yield [11]. Next, we synthesized the aldehyde γ-bicyclohomofarnesal (**26**) from commercially available (+)-sclareolide. Thus, (+)-sclareolide was reacted with the dimethylaluminum amide derived from *N*-methoxy-*N*-methylamine yielding the desired Weinreb´s amide **24** in 85% yield (Scheme 2). The tertiary alcohol of amide **24** was dehydrated in the presence of $SOCl_2$/pyridine at -78ºC to give essentially the *exo*-isomer **25** in 90% yield. Finally, compound **25** was treated with DIBAL-H



Reagents: a) MOMCl, K_2CO_3; b) Me_2SO_4, LiOH; c) $LiAlH_4$, THF; d)PCC/AI_2O_3; e) MeONHMe.HCl, $AIMe_3$; f) $SOCl_2$/py; DIBAL-H; h) Dess-Martin periodinane.

Scheme 2: Synthesis of aldehydes **20**, **23**, **26**, and **28**.

at -78ºC to give the desired aldehyde **26** in 92% yield [12]. 3-Pyridinepropanol **27** was oxidized with Dess-Martin periodinane to give the desired 3-pyridinepropanal **28** in 96% yield. Commercial furfural (**7**) was used as received without purification.

With the aldehydes **7**, **20**, **23**, **26**, and **28** in hand, our next objective was to accomplish the key Wittig reaction to introduce the 3,4,5-trimethoxybenzene and *cis*-olefinic bridge moieties. The necessary ylide derived from phosphonium salt **18** was generated with butyllithium in THF at -30ºC prior to the addition of the different aldehydes at this temperature and then allowing the mixture to warm to room temperature slowly. All reactions worked giving the corresponding *cis*-stilbenes **9-11** and MOM-protected CA-4, after careful silica gel column chromatography, with good yields (65-90%) and *Z:E* (ratio ca. 2:1 to 4:1) selectivity, except for the reaction with abietinal **23** to afford **8** that failed, probably due to steric hindrance present in the reacting aldehyde group. Combretastatin A-4 (**1**) was obtained after deprotection of the MOM-protected molecule with refluxing acetic acid in water (8:2) (90%). Compounds **1** and **11** are previously known [1,19], while **9** and **10** are new.

The combretastatin-based hybrids **9-11**, combretastatin A-4 (**1**) itself, and compounds **12-14** were tested *in vitro* for potential antitumor and cytotoxic activities by determining the concentration of the compound that induces 50% growth inhibition (IC_{50}) on the HeLa tumor cell line and non-tumor Vero cell line, respectively (Table 1). The antitumor activity on HeLa cells, and cytotoxic activity on Vero cells have been carried out using the tetrazolium-dye (MTT) assay *in vitro* on cell growth according to the protocol reported by us [20], which was used with a few modifications. Vincristine was used as positive control. All these compounds produced a dose-dependent inhibition of the growth of cell lines, with R^2 (coefficient of linear regression) > 0.8. The derivatives **1, 11** and **12** showed *in vitro* potential antitumor activity. The combretastatin-hybrid **11** with a furan ring showed the highest potential antitumor activity on the HeLa tumor line with an IC_{50} value of 3.0 ± 0.5 μg/mL. However, the compound with the highest selectivity index (SI) was pyrogallol (**12**). The susceptibility of tumor cell lines to pyrogallol has also been exhibited for A549 lung cancer cells and its non-cytotoxicity to MRC-5 normal lung fibroblasts [21]. Selectivity refers to the ability of a compound to

Table 1: Cytotoxic activity of compounds **1**, **9-14** determined by the MTT technique expressed as IC_{50} (μg/mL).[a]

Compound	Cell line				
	HeLa[b]		Vero[c]		
	CC_{50}	$R^{2,d}$	IC_{50}	R^2	SI[e]
1	6.3±3.3	0.7	30.7±0.7	1.0	4.9
9	15.1±1.6	0.9	26.4±5.3	0.7	1.8
10	15.9±1.9	0.9	106.2±3.9	1.0	6.7
11	3.0±0.5	0.8	28.9±5.2	0.8	9.6
12	4.1±1.2	0.8	120.0±12.6	0.9	29.4
13	21.3±1.1	1.0	39.9±4.3	0.9	1.9
14	39.6±5.2	1.0	>200	--	>5.05
Vincristine	0.05±0.01	1.0	1.1±0.2	1.0	22

[a] Concentration of compounds which induces 50% growth inhibition in 48 h. [b] HeLa, human cervix epithelial carcinoma; [c] Vero, *Cercopithecus aethiops* African green monkey kidney; [d] R^2: coefficient of linear regression. [e] SI, selectivity index is defined as Vero IC_{50} over HeLa IC_{50}.

recognize its target without interacting with other targets. Recently, an SI value of 14.3 on cell lines is indicative of potential antitumor activity for biopharmaceutical use [22]. Therefore, the combretastatin- hybrid **11** with a SI value of 9.63 could be a possible antitumor agent.

All compounds **1**, **9-14** were tested for anti-*Candida*, anti-*Aspergillus*, anti-*Fusarium* and anti-dermatophyte activity. The antifungal activity was evaluated following the standard method proposed by the Antifungal Susceptibility Testing Subcommittee of the European Committee on Antibiotic Susceptibility Testing (AFST EUCAST) [23] for fermentative yeasts and the Clinical and Laboratory Standards Institute M38-A (CLSI M38-A, 2002) protocol [24] for filamentous fungi, which was used with a few modifications. The yeast *C. parapsilosis* (ATCC 22019), *C. krusei* (ATCC 6258), *C. tropicalis* (CECT 11901), *C. albicans* (ATCC 10231) and the filamentous fungi *Aspergillus fumigatus* (ATCC 204305), *A. flavus* (ATCC 204304), *A. terreus* (CDC 317), *Fusarium oxysporum* (ATCC 48112), and the dermatophytes *Trichophyton rubrum* (ATCC 28188) and *T. mentagrophytes* (ATCC 24198) were used to evaluate antifungal activity. Amphotericin B (AMB), itraconazole (ITZ) and terbinafine (TERB) were used as positive controls with *Candida* spp., *Aspergillus* spp. and dermatophytes species, respectively, where the MIC values for the three reference drugs were within the established values for the standard protocols (Table 2). The compounds did not show activity against *Candida parapsilopsis, C. krusei, C. tropicalis, C. albicans*, *Aspergillus flavus* and *Fusarium oxysporum* at concentrations lower than 100.0 μg/mL, except the pyrogallol compound **12** that showed MIC values of 6.25 ± 0 μg/mL, 25.0 ± 0 μg/mL, 79.4 ± 25.8 μg/mL, 100 ± 0 μg/mL, 396.9 ± 129.1 μg/mL and 79.4 ± 28.9 μg/mL, respectively (data not shown). Moreover, the combretastatin-based hybrid **9** and compound **14** did not show activity against any fungi evaluated (Table 2).

Studies of antifungal activity against *Candida*, *Aspergillus*, *Fusarium* and dermatophyte species of pyrogallol have not been reported so far. Furthermore, in previous studies, Pettit *et al.*[25] did not find anti-*Candida* activity for combretastatin A-4 (**1**), however, a combretastatin-hybrid with a nitroethylene moiety, the 3,4,5-trimethoxy-β–nitrostyrene, showed activity against *C. albicans* ATCC 90028 (MIC = 32.0 μg/mL). In this study, pyrogallol (**12**) also showed MIC values of 396.8 ± 129.1 μg/mL against *A. fumigates,* 31.5 ± 14.4 μg/mL against *A. terreus* and 25 ± 0 μg/mL against *T. rubrum* and *T. mentagrophytes*. In addition, gallic acid (**13**) had activity against *T. rubrum* and *T. mentagrophytes* (MIC = 50.0 ± 0 μg/mL and 39.7± 12.9 μg/mL, respectively). Moreover, the combretastatin-based hybrid **11** and combretastatin A-4 (**1**) showed MIC values much lower than those of compounds

Table 2: Antifungal activity of compounds **1**, **9-14** expressed as MIC values in μg/mL[a]

Compound	GM-MIC (μg/mL)[a]			
	A. fumigatus (ATCC 204305)	*A. terreus* (CDC 317)	*Trichophyton rubrum* (ATCC 28188)	*Trichophyton mentagrophytes* (ATCC 24198)
1	50 ± 0	>100	19.8 ± 6.5	15.8± 6.5
9	>100	>100	>100	>100
10	50 ± 34.2	>100	100 ± 0	50± 0
11	19.8 ± 6.5	63 ± 25.8	15.7± 6.8	15.8± 6.5
12	396.9 ± 129.1	31.5 ± 14.4	25 ± 0	25 ± 0
13	>100	>100	50 ± 0	39.69± 12.91
14	>100	>100	>100	>100
TERB	--	--	<0.078 ± 0	0,03125 ± 0
AMB	2 ± 0	-	-	-
ITZ	0.17 ± 0.1	--	--	--

[a] Geometric means of minimal inhibitory concentration of tested compounds in μg/mL.

Table 3: Antiviral activity: Cytotoxicity and anti-HHV-1/ HHV-2 activity of **1**, and **9-14** on Vero Cells **determined by the end-point titration technique.

Compound	HHV-1			HHV-2	
	CC_{100} (μg/mL)[a]	Viral Reduction Factor[b]	Antiviral Activity (μg/mL)[c]	Viral Reduction Factor[b]	Antiviral Activity (μg/mL)[c]
1 (39)	100	$10^{0.5}$	50	10^2	25
9 (40)	25	$10^{0.5}$	12.5	10^2	3.125
10 (47)	>50	$10^{0.5}$	50	N.A	N.A
11 (43)	50	N.A	N.A	N.A	N.A
12	>50	N.A	N.A	N.A	N.A
13 (44)	>50	10^1	6,25	10^2	6,25
14 (41)	>200	N.A	N.A	N.A	N.A
Heparin (I.U/mL)	>100	10^2	0.5	10^2	0.5
Acyclovir	> 600	10^4	6.0	10^4	6.0

[a] Minimal toxic dose that detached 100% of the cell monolayer. [b] ratio of the virus titer in the absence over virus titer in the presence of the tested compound. [c] maximal nontoxic dose that showed the highest viral reduction factor. N.A: no activity.

12 and **13** against *A. fumigatus* and the dermatophytes *Trichophyton rubrum* and *T. mentagrophytes*, the hybrid **11** showing the highest activity in all cases (MIC = 19.8 ± 6.5 μg/mL, 15.7 ± 6.7 μg/mL and 15.7 ± 6. 4 μg/mL, respectively). Furthermore, only the combrestastatin-hybrid **11** showed activity against *Aspergillus terreus* with an MIC value of 63.0 ± 25.8 μg/mL. It is important to note that *Aspergillus fumigatus* is the most important mold causing fungemia and is known to have high resistance to many antimycotic agents. On the other hand, dermatophytes are the most common etiological agents of the superficial fungal infection known as dermatophytosis, which affects skin, hair and nails. To the best of our knowledge, this is the first report of antifungal activity on *Aspergillus* spp. and dermatophyte species of combretastatin A-4 (**1**), combrestastatin-based hybrids, and gallic acid derivatives.

The antiviral activity of compounds **1**, **9-14** against Herpes simplex virus type 1 (HHV-1), obtained from the Center for Disease Control (Atlanta, GA) and HHV-2 (ATCC VR-734, strain G) was carried out on Vero cells using an end-point titration technique, according to the protocol reported by us [26], which was used with a few modifications [27]. Combretastatin A-4 and hybrid **9** showed activities against HHV, and reduced HHV-2 replication with concentrations below 25.0 and 3.1 μg/mL, respectively (Table 3). Gallic acid (**13**) showed the highest antiviral activity for HHV-1 and HHV-2.

According to Vlietinck *et al.* [28], only compounds with a reduction factor (*Rf*) of the viral titer over 1 x 10^3 (Rf: ratio of the virus titer in the absence over virus titer in the presence of the tested compound) show relevant antiviral activity. Gallic acid (**13**) was found to be slightly active against HHV-1 and HHV-2 over infected confluent monolayers of Vero cells with an *Rf* value of 1x10^1 and 1x10^2 at a concentration of 6.25 μg/mL. Kratz *et al.* [29] have reported the activity of gallic acid and pentyl gallate against HHV-1 and HHV- 2

[29,30]. This is the first time that anti-herpetic activity of combretastatin A-4 (**1**) and combrestastatin-based hybrids is described.

Experimental

Chemistry: Optical rotations were determined using a 5 cm path-length cell, using dichloromethane as solvent (concentration expressed in g/100 mL). $[\alpha]_D$-Values are given in 10^{-1} deg cm^2/g. NMR spectra were recorded on a 300 MHz spectrometer with tetramethylsilane as an internal standard. All spectra were recorded in $CDCl_3$, unless otherwise described. Complete assignments of ^{13}C NMR multiplicities were made on the basis of DEPT experiments. *J* values are given in Hertz. Mass spectra (MS) were run by electron impact (EI) at 70 eV. Reactions were monitored by TLC using Merck silica gel 60 F-254 on 0.25 mm thick plates. Compounds on TLC plates were detected under UV light at 254 nm and visualized by immersion in a 10% sulfuric acid solution and heating on a hotplate. Purifications were performed by flash chromatography on Merck silica gel (230–400 mesh). All non-aqueous reactions were carried out in an argon atmosphere in oven-dried glassware. Commercial reagent grade solvents and chemicals were used as received unless otherwise noted. Combined organic extracts were washed with brine, dried over anhydrous sodium sulfate, filtered and concentrated under reduced pressure.

All compounds gave satisfactory spectroscopical NMR data:

Compound 1

^1H NMR (300 MHz, $CDCl_3$) δ: 6.90 (1H, d, *J* = 1.8), 6.77 (1H, dd, *J* = 8.1, 1.8), 6.70 (1H, d, *J* = 8.4), 6.51 (2H, s), 6.44 (1H, d, *J* = 12.3), 6.37 (1H, d, *J* = 12.3), 5.80 (1H, br s), 3.82 (3H, s), 3.81 (3H, s), 3.66 (6H, s);
^{13}C NMR (75 MHz, $CDCl_3$) δ: 152.8 (2 C), 145.8, 145.2, 137.1, 132.7, 130.6, 129.5, 128.9, 121.1, 115.1, 110.4, 106.0 (2 C), 60.9, 55.9 (3 C).

Compound 9

^1H NMR (300 MHz, $CDCl_3$) δ: 6.43 (2H, s), 6.17 (1H, d, *J* = 11.8), 5.44 (1H, ddd, *J* = 12.3, 6.6, 5.7), 4.71 (1H, s), 4.29 (1H, s), 3.75 (9H, s), 0.77 (3H, s), 0.71 (3H, s), 0.61 (3H, s).
^{13}C NMR (75 MHz, $CDCl_3$) δ: 152.5 (2 C), 147.9, 136.4, 133.3, 132.9, 128.0, 107.6, 105.6 (2 C), 60.5, 57.2, 55.7 (2 C), 55.1, 41.8, 39.2, 39.1, 37.8, 33.3, 33.2, 23.9, 23.6, 21.5, 19.1, 14.3.

Compound 10

^1H NMR (300 MHz, $CDCl_3$) δ: 8.47 (2H, m), 7.51 (1H, m), 7.21 (1H, m), 6.56 (1H, s), 6.55 (1H, s), 6.17 (1H, m), 3.85 (6H, s), 3.84 (3H, s), 2.78 (2H, m), 2.52 (2H, m).
^{13}C NMR (75 MHz, $CDCl_3$) δ: 153.1 (2 C), 152.7, 149.8, 136.6, 135.7, 133.0, 130.8, 128.3, 123.1, 105.5, 102.8 (2 C), 60.7, 55.8 (2 C), 34.2, 32.7.

Compound 11

^1H NMR (300 MHz, $CDCl_3$) δ: 7.33 (1H, br s), 6.74 (2H, s), 6.39 (1H, d, *J* = 12.8), 6.32 (1H, d, *J* = 12.6), 3.88 (3H, s), 3.83 (6H, s).
^{13}C NMR (75 MHz, $CDCl_3$) δ: 153.3, 152.8 (2 C), 151.9, 141.5, 132.6, 127.8, 117.4, 111.3, 110.4, 105.8, 103.2, 60.9, 55.9 (2 C).

MTT Assay: The cells used were: Vero (African green monkey kidney, ATCC: CCL 81 line) and HeLa (human cervix epithelial carcinoma, ATCC CCL-2) cell lines. Initially, cells were kept in log phase of growth in MEM modified by Dulbecco (DMEM) supplemented respectively with 5% fetal bovine serum (SBF), 1μg/mL penicillin, streptomycin 1μg/mL, 1μg/mL of neomycin, vitamins, nonessential amino acids and 1% glutamine. The pH of 7.2 required for cell cultures was achieved with 1N sodium hydroxide, and stabilized with 0.5% aqueous sodium bicarbonate solution, 7%. Cell cultures were maintained at 37°C in a humidified 5% CO_2 atmospheric chamber. Then, cell monolayers were trypsinized and washed with culture medium and then plated at 1.5 x 10^4 cells per well for HeLa and 1.25 x 10^4 cells per well for Vero cells in 96-well flat-bottomed plates. After 24 h of incubation, each diluted compound was added to the appropriate wells and the plates were incubated for a further 48 h at 37°C in a humidified incubator with 5% CO_2. Vincristine and pyrogallol were used as positive controls. The CC_{50} for each compound was obtained from dose–effect curves by linear regression methods and CC_{50} values are expressed as the mean ± S.E.M. of least 4 dilutions, repeated 8 times.

Antifungal activity: The standard methods proposed by the Antifungal Susceptibility Testing Subcommittee of the European Committee on Antibiotic Susceptibility Testing (AFST EUCAST) [23] for fermentative yeasts and the Clinical and Laboratory Standards Institute M38-A (CLSI M38-A, 2002) [24] for filamentous were used with a few modifications. The dilutions of the compounds were dispensed into 96-well flat-bottom microdilution plates in duplicate at final concentrations between 100.0 μg/mL and 2.0 μg/mL. Amphotericin B (Sigma Chemical Co, MO, USA) with *A. fumigatus* and *A. flavus*, itraconazole (Sigma Chemical Co, MO, USA) with *C. krusei* and *C. parapsilosis* and terbinafine (Recalcine Laboratories, Santiago de Chile, Chile) with dermatophytes were used as positive controls at a range of 0.031-16.0 μg/mL. The inoculum sizes for microdilution plates were 0.5 - 2.5 x 10^5 and 0.2- 2.5 x 10^5 CFU/mL for yeast and filamentous fungi, respectively. For the AFST-EUCAST method, the Minimal Inhibitory Concentrations (MICs) were determined after 24 h at 35°C of incubation by spectrophotometric reading at 405 nm and is defined as the lowest concentration that resulted in a 90% reduction of growth. For the CLSI M38-A method, the MICs were determined after 48 h of incubation at 35°C for *Aspergillus* spp. and at 28°C for *F. oxysporum*. The activity against the dermatophytes was determined after 6 days of incubation at 28°C. The MICs were defined as the lowest dilution that resulted in 80% inhibition of visible growth. MIC results were expressed as range and geometric mean (GM) of duplicates of each compound tested 3 times against each of the fungal species in different assays.

Antiviral activity: Herpes simplex virus types 1 and 2 were obtained from the Center for Disease Control (Atlanta, GA). Virus stocks (HHV-1 and HHV-2) were prepared from infected Vero cell cultures using an end-point titration technique. Two-fold dilutions of the compounds and viral suspension (one infection dose, 1 D.I.) were mixed and incubated for 0.5 h at 21°C before they were added onto confluent monolayer cells and incubated again at 37°C in a humidified 5% CO_2 atmosphere for 36 h. Acyclovir and heparin sodium salt were used as positive controls.

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