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SAR studies of epoxycurcuphenol derivatives on leukemia CT-CD4 cells

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ABSTRACT

Bioactive natural products are a potential source of new pharmaceuticals since they offer new modes of action and more specific activities. The use of derivatization also enables the optimal structure for their biological activity to be determined. In this study several epoxycurcuphenol derivatives were synthesized. The substitution pattern on the aromatic and oxirane rings was varied along with that at the benzylic position and the length of the side chain was altered. These changes were made in order to gain a deeper understanding of the structural requirements for activity. The biological activities of these compounds were evaluated on the human leukemia cell line Jurkat using an antiproliferative assay. The activity results and structural requirements are discussed.

1. Introduction

Bioactive natural products are a potential source of new pharmaceuticals[1,2] since they offer new modes of action and more specific interactions. Among the plants and microorganisms from which allelopathic agents have been isolated, *Helianthus annuus* L. (sunflower) is very interesting because of its high production of secondary metabolites[3] especially in sesquiterpene lactones,[4,5] heliespirones,[6,7] annuionones,[8] helibisabonols,[9] heliannuols[10,11] and phenolic compounds.[12]

Heliannuols, a new class of compounds isolated from *Helianthus annuus*, constitute a family of new compounds with a novel heterocyclic sesquiterpene structural backbone named heliannane.[13] This skeleton is characterized by a benzenoid moiety fused to a five- to eight-membered heterocyclic ring. Among these compounds, heliannuol D (**1**) (Fig. 1) has special relevance due to the high phytotoxic activity it has shown.[14] The fact that these allelochemicals may be useful for developing new active compounds makes their synthesis at large scale an attractive goal.[15] Besides the synthetic analogous and intermediates obtained during their synthesis are also of interest, since their bioactivity offers hints about the structural requirements needed.

In the course of our research toward the synthesis of heliannuol D (**1**)[16] we obtained two aromatic diasteroisomers that present an oxirane group (**2a**, **2b**) (Fig. 1). They showed interesting biological activities when evaluated in animal cells. Compounds **2a** and **2b** were evaluated for their effects on cytokine production by human primary CD4+ T cells and on cell division on the human tumoral cell line Jurkat, murine antigen specific CD4+ T cell line D5, murine Primary CD4 T Cells from TcR (T cell receptor) Transgenic DO11.10 mice, human primary naïve PBMC and human primary CD4+ T cells. In the case of the lymphoid human cell line Jurkat, addition of sesquiterpenes at a final concentration of 1 μM impaired cell division in almost 100% of cells.[17]

In order to gain a deeper understanding of the structural requirements for bioactivity in this family of compounds we carried out a Structure–Activity Relationship (SAR) study and

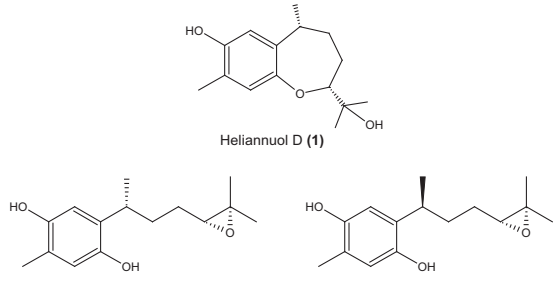

Figure 1. Structures of heliannuol D and its intermediates with an oxirane ring **2a** and **2b**.

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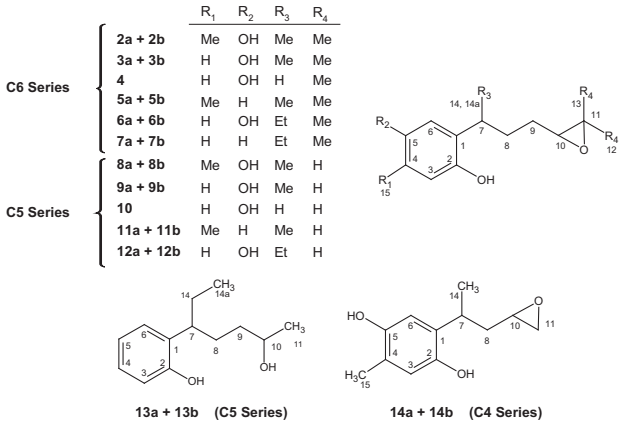
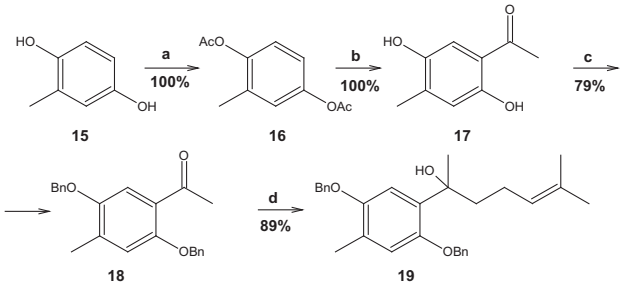


Figure 2. Compounds synthesized. Numbering of compounds are assigned as derivatives of curcuphenol or curcuhydroquinone, in agreement with IUPAC recommendation in section F (see Supplementary data), so for **14a**/**14b** 9,12,13-trinor-curcuhydroquinone is assigned.

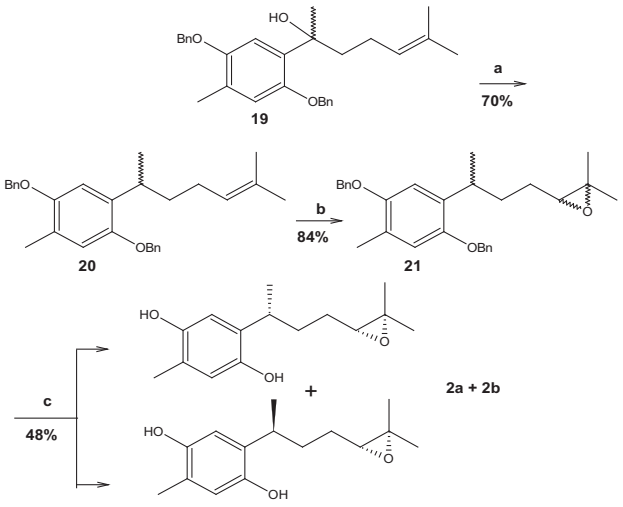
synthesized 24 derivatives with different side chains and substitution patterns in the aromatic ring and the alkylic side chain (Fig. 2). We changed the number and position of hydroxyl groups, the presence of additional methyl or ethyl groups in the aromatic ring and/or in the side chain and the pattern of substitution in the aromatic ring. The compounds were classified into three families depending on the length of the side chain and the substitution pattern at the oxirane ring: C6 (oxirane ring with two methyl groups in the last carbon, and a side chain that is six carbons in length; compounds **2**–**7**), C5 (with a side chain five carbons long and the oxirane ring without any terminal methyl groups; compounds **8**–**13**) and C4 (with a four-carbon side chain and an oxirane ring without any methyl group; compound **14**) (Fig. 2). All compounds were obtained as a pair of diastereoisomeric products apart from compounds **4** and **10**.

2. Chemistry

A total of 24 compounds were synthesized (Fig. 2); 18 of them were tested for antiproliferative potential (Jurkart cell line from leukemia CT-CD4) to evaluate different modifications on the basic skeleton of **2a** and **2b**. The synthesis is based on that of heliannuol D and resembles the biogenetic route proposed for heliannuols and helibisabonols, where compounds **2a** and **2b** are intermediates proposed in this biogenetic route.[10,11,18] Thus, the synthetic procedure used herein has two main stages. The first stage involves the preparation of the appropriate aromatic bisabolene skeleton **19** from a benzenoid precursor (2-methylhydroquinone **15** as starting material) with the desired substitution pattern in the aromatic ring



Scheme 1. (a) $(CH_3CO)_2O$, Py, 24 h, rt; (b) $BF_3{\bullet}Et_2O$, 3 h, 120 °C; (c) Benzyl Bromide, K_2CO_3, Dimethoxyethane, 12 h, 80 °C; (d) 5-Br-2-methyl-pentene, Mg, I_2, THF, 1 h, 65 °C.



Scheme 2. (a) Et_3SiH, $BF_3{\bullet}Et_2$, DCM, −55 °C → 0 °C, 12 h (b) *m*-CPBA, EtOAc, DCM, 2 h, rt; (c) H_2/Pd/C, DMF, 2 h, rt.

and the correct side chain obtained according the procedures reported by our group (Scheme 1).[16] The second stage of the synthesis (Scheme 2) hasthree steps and these are slightly different to those reported previously. The first step was the reduction of the hydroxyl group at C-7 (**20**) and this was achieved using triethylsilane in dichloromethane and $BF_3{\bullet}OEt_2$ as catalyst to give the desired product in 70% yield. Subsequent epoxidation of **20** gave compound **21** in 84% yield. Finally, palladium-catalyzed (Pd/C) hydrogenation of **21** led to selective cleavage of the benzylic ethers to yield the pair of diastereoisomers **2a** and **2b** in 45% yield each. These compounds could be separated by HPLC.

Depending on the nature of the starting material, this synthetic methodology led to the corresponding pairs of diastereoisomers shown in Figure 2. The intermediates are shown in the Supplementary data, although some reactions needed to be slightly modified depending on the specific reactivity requirements of the intermediates. For example, the dehydroxylation reaction to give the precursors of **4** and **10** required the use a large excess (10 equiv) of triethylsilane and the reaction was carried out at 0 °C. The epoxidation in the C5 series was performed with an increased amount of m-CPBA and a longer reaction time. All pairs of diastereoisomers were separated and purified by HPLC techniques, with the exception of **14a** and **14b**, which could not be isolated. In the hydrogenation reactionto obtain **13a** and **13b** it was impossible to remove the aromatic hydroxyl group without affecting the oxirane ring. In this case, the final compounds were those resulting from reductive cleavage of the oxirane ring. The absence of terminal methyl groups in the oxirane ring of compounds in C5 markedly changed the reactivity of the system and this is the probable cause of this behaviour.

3. Results and discussion

3.1. Synthesis

The synthetic procedure used for the pair of diastereoisomers **2a** and **2b** has been used and optimized to obtain the complete family of compounds presented. Only slight modifications were necessary in the synthesis of some compounds, with changes to the amount of reagent or the reaction temperature, as described in the Experimental section, to improve yields. All reactions proceeded with good yields (between 40 and 95%) (Supplementary data page S6).

3.2. Relative stereochemistry

The final compounds **2a**/**2b** to **14a**/**14b** have two chiral centres at C-7 and C-10 (in the C6 and C5 series) or C-7 and C-9 (in the C4 series) that provide two pairs of diastereoisomers each (except for **4** and **10**, which have one asymmetric centre). The relative stereochemistry of each diastereoisomer was elucidated by NMR and theoretical conformational studies that will be discussed below.

The structures of all compounds were determined by spectroscopic methods (^{1}H and ^{13}C NMR, MS and IR). There are some trends that are maintained for all diastereoisomers in each series. The less polar compound **2a** shows collapsed signals for protons H-8 (δ 1.67, td, J_{7-8} = 7.1, J_{8-9} = 7.9 Hz, 2H) and H-9 (δ 1.41, q, J_{8-9} = 7.9, J_{9-10} = 7.6 Hz, 2H). On the other hand, the more polar compound **2b** presents separate signals for each proton at C-8; H-8 (δ 1.75, m) and H-8′ (δ 1.59, ddt, $J_{8-8'}$ = 12.8, $J_{7-8'}$ = 9.7, $J_{8'-9}$ = 6.3 Hz) (Fig. 3). These facts could suggest an intramolecular bonding in one of the isomer, which influence its polarity.

Such differences suggest the presence of a rotational barrier through the C-8/C-9 bond where the two protons are differentiated. A careful study of the structure of compounds **2a**/**2b** shows that a hydrogen bond can be established between the aromatic hydroxyl group and the oxirane ring. Such a bond provides enough rigidity to differentiate the two H-8 signals.

A theoretical model was developed for each diastereoisomer using PCMODEL with GMMX (Global-MMX) calculations and MOPAC (Molecular Orbital PACkage)[19,20] to obtain the minimum energy conformers that contain a hydrogen bond. In the case of the 7*R**10*R** diastereoisomer two minimum energy conformers bearing the hydrogen bridge were found (Table 1), but none of these had dihedral angles with coupling constants similar to the experimental values. Two conformers were also found for the 7*S**10*R** isomer. In this case the theoretical coupling constants were similar to the experimental values. Consequently, a relative stereochemistry 7*S**10*R** is proposed for compound **2b** and a relative stereochemistry 7*R**10*R** for **2a**. The 3D representations of the selected conformers for each compound are shown in Figure 4, where the hydrogen bond formed between the aromatic hydroxyl group and the oxirane ring on **2b** can be observed. The same theoretical study was carried out for the rest of the compounds obtained, allowing us to propose the relative stereochemistries shown in Table 2.

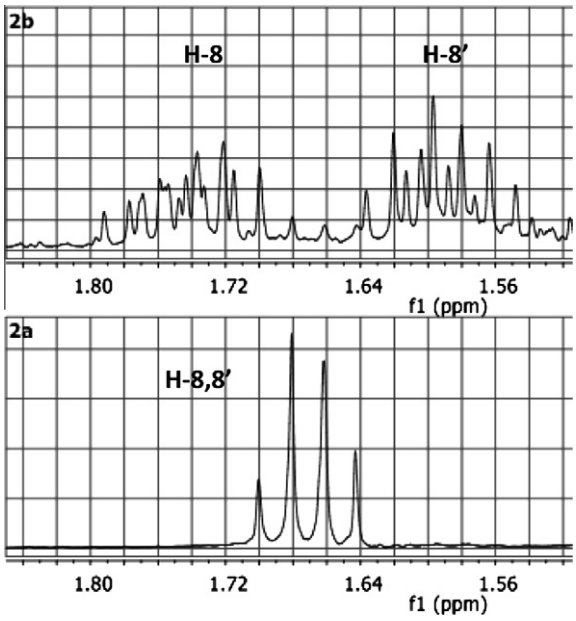


Figure 3. Detail of the ^{1}H NMR region for the protons at C-9 for **2a** (bottom) and **2b** (top).

3.3. Antiproliferative assay on human cells

In this bioassay, we analyse cell division as the end point of cell proliferation. Cells were exposed to test compounds at a concentration of 10 μM for 24 h, stained with CFSE (Carboxyfluorescein succinimidyl ester)[21–23] and analysed by flow cytometry 4 days later to determine size distribution and the level of cell division. The effect of the active compound can be detected from a shift in the population curve to the left when compared to the control curve. This change is due to dilution of the CFSE dye between dividing cells. The effect was quantified using a flow cytometer (Cyan ADP MLE ™, Bechman ™) to count dividing and non-dividing cells. Several parameters have been compared to show the activity of the compound in comparison with the control value, with the results expressed as the Proliferation Index (PI, a measure of the increase in cell numbers in the culture) and Nonproliferative Fraction (NpF, which represents the fraction in the original culture cells that have not proliferated during the course of the experiment). The results are shown in Table 3 and Figure 5.

Of the compounds tested, those with two aromatic hydroxyl groups are the most active. This effect is clear on comparing compounds that differ only in the number of aromatic hydroxyl groups; for example, in the C6 series, **2a** (NpF: 0.87) and **2b** (NpF: 0.92) are more active than **5a** (NpF: 0.41) and **5b** (NpF: 0.42), and the same effect can be observed with **8a** (NpF: 0.86) and **8b** (NpF: 098) when compared to **11a** (NpF: 0.37) and **11b** (NpF: 0.30) in the C5 series (Fig. 5).

The substituent at the benzylic position (C-7) also modifies the activity. In this case, the optimal group in this position is hydrogen, as shown by comparison of the bioactivities of compounds **3b**, **4** and **6b** (Table 3). Thus, **4** does not have a substituent at C-7 and it is the most active compound (NpF: 0.94), followed by **6b** (NpF: 0.62) and **3b** (NpF: 0.39), which have an ethyl and a methyl substituent, respectively. Other structural factors analysed were the substitution pattern of the oxirane ring, polarity of compounds and the relation with the stereochemistry and the presence or absence of an aromatic methyl group at C-4 position.

The substitution pattern on the oxirane ring affects the activity by enhancing the effect produced by the factors discussed above. In most cases, compounds of the C5 series without methyl groups on the oxirane ring show lower activities than compounds of the C6 series that contain two methyl groups at the end of the side chain on the oxirane ring as the only difference. Comparison of the activities of **5a**/**5b** (NpF: 0.42/0.41) with **11a**/**11b** (NpF: 0.37/0.30) or **2a** (NpF: 0.87, PI: 1.62) versus **8a** (NpF: 0.86, PI: 1.85)) illustrates this behaviour (Table 3). This trend is particularly pronounced in the comparison of the active compounds **6a**/**6b** (NpF: 0.59/0.62) with the inactive compounds **12a**/**12b** (NpF: 0.40/0.42). This behaviour can be due to the +I inductive effect of methyls over oxirane ring increasing the reactivity of this moiety and then their toxicity.[24]

For active compounds, the most polar one in every pair of diastereoisomers, corresponding to the relative stereochemistry 7*S**,10*R** (Table 2), is also the most active. Thus, **8b** (PI: 1.09; NpF: 0.98) is more active than **8a** (PI: 1.85; NpF: 0.86) and **2b** (PI: 1.36; NpF: 0.92) is more active than **2a** (PI: 1.62; NpF: 0.87) (Fig. 5).

The aromatic methyl group is another requirement for activity in this kind of compound. All compounds that do not bear an aromatic methyl at the C-4 position (**3b**, **4**, **6a**, **6b**, **7a**, **7b**, **12a**, **12b**, **13a**, **13b**) are inactive with the exceptions of **4** (PI: 1.11; NpF: 0.94) and **6a**/**6b** (PI: 2.73/3.04; NpF: 0.59/0.62). These values, which are very similar to those of the control and have a high population percentage in the 4th and 5th generation, are indicative of low activity. The activity of **4** is due to the fact that other structural

Table 1
Conformers with hydrogen bridge obtained for 7*R**10*R** and 7*S**10*R** relative stereochemistry of diastereoisomers 2a and 2b

Conformer	ΔH_f	Dihedral angle		Coupling constants		d_{OH-O}	μ_{dip}
		$\Phi_{7,8}$	$\Phi_{9,10}$	$J_{7,8}$	$J_{9,10}$		
		$\Phi_{7,8'}$	$\Phi_{9',10}$	$J_{7,8'}$	$J_{9',10}$		
7R*10R*#1	−117.85	−66.91°	59.68°	2.29	2.20	1.63	3.648
		177.11°	175.33°	12.32	11.66	7.63	
7R*10R*#2	−117.48	−66.95°	59.75°	2.29	2.20	1.63	2.435
		177.06°	175.42°	12.32	11.65	8.02	
7S*10R*#1	−113.00	161.02°	154.20°	11.12	10.63	1.99	4.357
		−83.14°	40.49°	1.05	4.86	7.93	
7S*10R*#2	−113.25	−51.14°	−179.48°	4.32	11.49	1.63	3.687
		62.98°	65.80°	2.57	11.6	7.86	

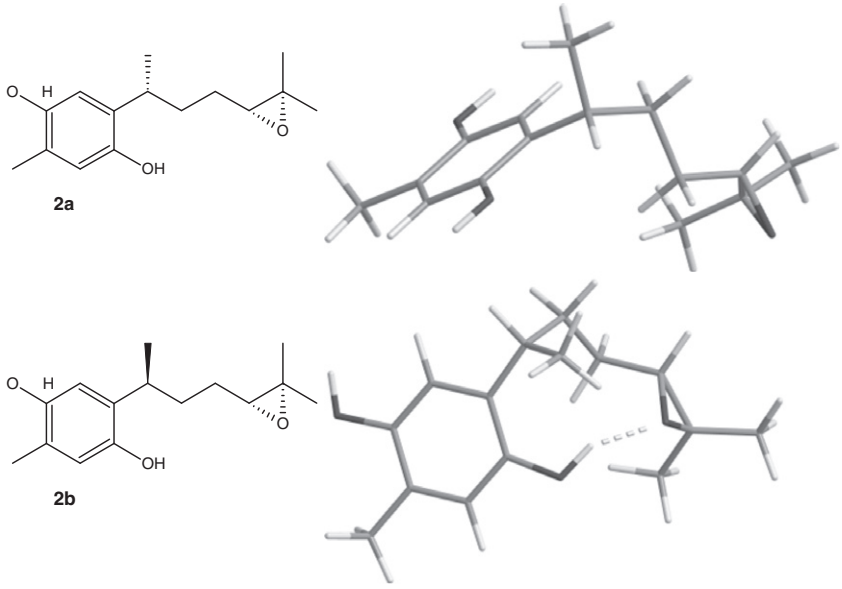


Figure 4. Relative stereochemistry and conformer proposed for the diastereoisomers **2a** and **2b**.

Table 2
Relative stereochemistry propose for each couple of diastereoisomers

Diastereoisomers	Stereochemistry	Diastereoisomers	Stereochemistry
2a/2b	7R*10R*/7S*10R*	8a/8b	7R*10R*/7S*10R*
3a/3b	7R*10R*/7S*10R*	9a/9b	7R*10R*/7S*10R*
5a/5b	7S*10R*/7R*10R*	11a/11b	7R*10R*/7S*10R*
6a/6b	7R*10R*/7S*10R*	12a/12b	7S*10R*/7R*10R*
7a/7b	7S*10R*/7R*10R*	13a/13b	7S*10R*/7R*10R*

requirements outlined below, for example two aromatic hydroxyl groups and/or the absence of a substituent at the benzylic position should have more relevance. Thus, the most active diastereoisomeric pairs are **2a**/**2b** and **8a**/**8b**, all of which have an aromatic methyl at C-4. Evermore, if we compare the activity of **2b** (NpF: 0.92) and **3b** (NpF: 0.399) with a methyl group on C-4 as only difference in their structure, we can determine that this substitution has an important influence on the activity of the compounds.

The most effective compound in this bioassay was 7*S**,10*R**-12,13-dinor-10,11-epoxy-10,11-dihydrocurcuhydro-quinone (**8b**), which fits the requirements described. Its effect can be shown with the shift in the population curve to the left when compared to the control curve (Fig. 6).

4. Conclusions

We have developed a facile synthetic route to epoxycurcuphenol derivatives that contain two chiral centres. These compounds

Table 3
Activities in antiproliferative assay with reference to control values

		PI	NpF	Parent (%)	Gen/%
Active compounds	2[a]	1.62	0.87	53.81	4/13.90
	2[b]	1.36	0.92	67.44	3/17.04
	4	1.11	0.94	84.84	2/7.31
	6[a]	2.73	0.59	21.49	3/35.78a
	6[b]	3.04	0.62	20.30	3/21.30b
	8[a]	1.85	0.86	46.39	5/18.91
	8[b]	1.09	0.98	89.59	3/6.73
	Control	2.48	0.59	23.70	4/25.99
Non active compounds	3b	6.47	0.39	6.02	5/64.37
	5[a]	4.77	0.41	8.71	5/38.84
	5[b]	5.20	0.42	8.09	5/49.30
	7[a]	4.10	0.48	11.75	5/29.37
	7[b]	4.38	0.46	10.46	5/33.67
	11[a]	5.86	0.37	6.23	5/47.87
	11[b]	5.71	0.30	5.27	5/40.06
	12[a]	5.06	0.40	7.95	5/48.54
	12[b]	5.01	0.42	8.46	5/47.67
	13[a]	5.52	0.34	6.10	5/40.57
	13[b]	6.11	0.32	5.19	5/46.79

PI: Proliferation Index.
NpF: Nonproliferative Fraction.
Gen:Most populated generation.
[a] The values for the cell population for the 4th and 5th generations are not insignificant, 13.90 and 11.80, respectively.
[b] Although the 3rd generation is the most popular, the fraction in the 4th has similar values (20.47) and slightly lower values (18.48) for the 5th generation, which are very close to the control values (25.99 and 18.43, respectively).

are obtained as pairs of diastereoisomers and their relative stereochemistries were determined by NMR spectroscopy and theoretical molecular studies.

We have studied the structure–activity relationship (SAR) in epoxycurcuphenols using the results from the antiproliferative potential in animal cells; it was possible to determine the structural requirements for optimal activity.

The main requirements for activity are: The compounds should have two aromatic hydroxyl groups without any substitution at the C-7 position. Moreover, the activity is improved when the relative stereochemistry is 7*S**,10*R**, a methyl group is present in the aromatic C-4 position and an monosubstituted oxirane ring is present. The most active compound synthesized was 8b (7*S**, 10*R**-12,13-dinor-10,11-epoxy-10,11-dihydro-curcuhydroquinone) and this has excellent Proliferation Index and Nonproliferative Fraction values (1.09 and 0.98, respectively).

In relation to the compounds that showed significant activity against animal cells, it would be of great interest to carry out further studies into phenomenon by making slight modifications to the structure and testing the resulting compounds in other bioassays in an effort to identify their mode of action.

5. Experimental

5.1. Chemistry

5.1.1. General

Commercially available chemicals were used as received. Dry THF was obtained by distillation of sodium-treated commercial THF. ^{1}H and ^{13}C NMR spectra (400 and 100 MHz, respectively) were recorded on a Varian Unity 400 NMR spectrometer with a sample temperature of 25 °C using $CDCl_3$ and $(CD_3)CO$. Mass spectroscopy was carried out using a GC-MS VG1250 apparatus (ion trap detector) in EI mode. FTIR (Fourier transform infrared), spectra were recorded on a Mattson 5020 spectrophotometer. Elemental analysis was carried out on a LECO CHNS analyser. Purities of synthesized compounds were determined by NMR and HPLC methods, and corroborated by HRMS with a degree of purity between 95–99%. For flow cytometry analysis, 10,000 live events were collected on a Cyan-ADP-MLE II flow cytometer (DakoCytomation™). Acquisition and analysis was performed using summit software (DakoCytomation™).

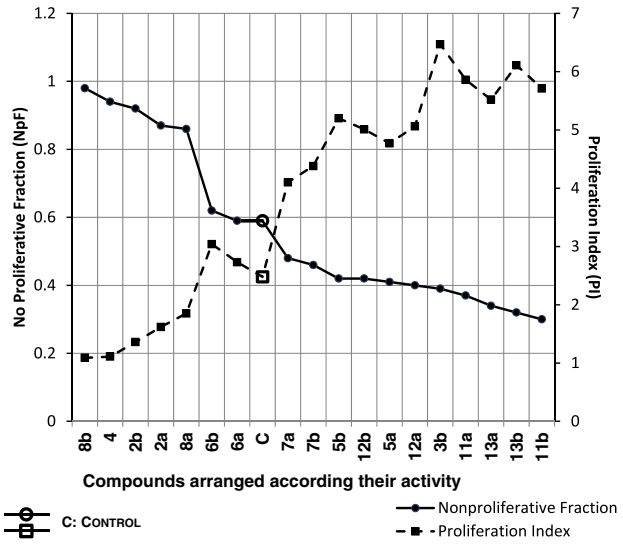


Figure 5. Antiproliferative bioassay results showing NonProliferative Fraction (**NpF**) and Proliferative Index (**PI**), the activity increases from left to right.

5.1.2. Synthesis of compounds 2a and 2b

5.1.2.1. 2,5-Diacetoxytoluene (16). This compound was obtained in quantitative yield without the need for chromatographic purification; IR (film, cm^{-1}); ^{1}H NMR ($CDCl_3$, 400 MHz, δ ppm); ^{13}C NMR ($CDCl_3$, 100 MHz, δ ppm) and HRMS data are consistent with that those reported previously.[16]

5.1.2.2. 2,5-Dihydroxy-4-methylacetophenone (17). This compound was obtained in quantitative yield, without the need for further purification, by the methodology reported previously;[16] the IR, ^{1}H NMR,^{13}C NMR and HRMS data are identical to those published previously.

5.1.2.3. 2,5-Dibenzyloxy-4-methylacetophenone (18). The crude product was purified by column chromatography (hexane/EtOAc 20%) to yield 2,5-dibenzyloxy-4-methylacetophenone (**18**) (95%). The product has identical spectroscopic data to those reported previously (IR, ^{1}H and ^{13}C NMR, HRMS).[16]

5.1.2.4. 2-*O*,5-*O*-Dibenzyl-7-hydroxy-curcuhydroquinone (19). A catalytic amount of I2 dissolved in dry THF (1 mL) was added under a N2 atmosphere to a dry flask with calcined magnesium (2 equiv). A solution of the aromatic compound **18** and 5-bromo-2-methyl-2-pentene (3 equiv) in dry THF (0.075 M in compound **18**) was added slowly with continuous stirring. The colour of the reaction change from muddy red to clear yellow and after 10 min the reaction was quenched with NH4Cl (sat) and extracted with AcOEt (×5). The combined organic layers were dried over anhydrous Na_2SO_4 and the solvent was evaporated under vacuum. The reaction mixture was separated by column chromatography (hexane/ethyl acetate 95:5). Compound **19** was obtained in 89% yield and the spectroscopic data are identical to those published by Macias et al.[16]

5.1.2.5. 2-*O*,5-*O*-Dibenzyl-curcuhydroquinone (20). Et_3Si (1 equiv) and $BF_3{\bullet}Et_2$ (0.5 equiv, dropwise) were added to 500 mg of 19 in DCM (0.02 M) under a N_2 atmosphere and the

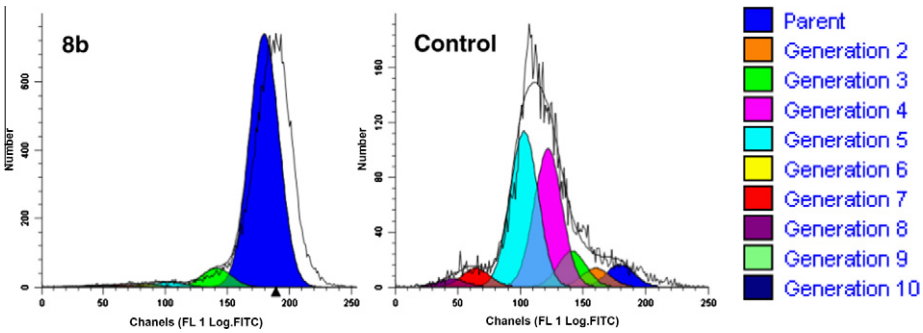


Figure 6. Population curves for the most active compound (**8b**) vs control, the cell division with **8b** is virtually nonexistent.

mixture was stirred at −78 °C. After 15 min the temperature was raised to −55 °C and the reaction mixture was stirred for 12 h under these conditions. The reaction was quenched with a saturated solution of $NaHCO_3$ and extracted with DCM (×3). The organic layer was dried over anhydrous Na_2SO_4, filtered and the solvent was evaporated. The mixture was purified by column chromatography (hexane/ethyl ether 95:5). Compound **20** was obtained in 70% yield. IR (film, cm^{-1}) ν_{max}: 1671, 1193, 859; ^1H NMR ($CDCl_3$, 400 MHz): δ 1.20 (d, 3H, H-14), 1.51 (dq, 1H, $J_{7,8/8'}$ = 7.0, $J_{8,8'}$ = 6.7, $J_{9,8/8'}$ = 7 Hz, H-8′), 1.54 (s, 3H, H-13), 1.62 (m, 1H, H-8), 1.67 (s, 3H, H-12), 1.89 (dt, 1H, $J_{9,10}$ = 7.0, $J_{9,8/8'}$ = 7.0 Hz, H-9), 2.28 (s, 3H, H-15), 3.25 (tq, 1H, $J_{7,8/8'}$ = 7.0, $J_{7,14}$ = 7.0 Hz, H-7), 4.99 (s, 2H, H-7″), 5.02 (s, 2H, H-7′), 5.08 (t, 1H, $J_{9,10}$ = 7.0 Hz, H-10), 6.75 (s, 1H, H-6), 6.77 (s, 1H, H-3), 7.38 (d, 2H, $J_{3',4';3'',4''}$ = 7.8 Hz, H-4′, H-4″), 7.40 (ddd, 4H, $J_{3',4';3'',4''}$ = 7.8, $J_{2',3';2'',3''}$ = 7.5, $J_{3',5';3'',5''}$ = 1.8 Hz, H-3′, H-5′, H-3″, H-5″), 7.45 (d, 4H, $J_{2',3';2'',3''}$ = 7.5 Hz, H-2′, H-6′, H-2″, H-6″); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 16.2 (C-15), 21.2 (C-14), 25.7 (C-13), 26.2 (C-9), 31.6 (C-7), 37.4 (C-8), 71.0 (C-7″), 71.1 (C-7′), 111.7 (C-6), 115.6 (C-3), 124.8 (C-10), 124.9 (C-11), 127.1 (C-2″, C-6″), 127.3 (C-2′, C-6′), 127.5 (C-1″), 127.6 (C-1′), 128.4 (C-3′, C-5′, C-3″, C-5″), 131.1 (C-4), 134.4 (C-4), 137.8 (C-4′, C-4″), 150.2 (C-5), 151.2 (C-2); HRMS calcd for $C_{29}H_{34}O_2$ 414.5791, found 414.2578. Elemental analysis calcd for $C_{29}H_{34}O_3$: C, 84.02; H, 8.27; O, 7.71, found: C, 84.79; H, 7.92; O,7.29.

5.1.2.6. 2-O,5-O-Dibenzyl-10,11-epoxy-10,11-dihydrocurcuhydroquinone (21). A slight excess of m-CPBA (1.12 equiv) and sodium acetate (1.2 equiv) were added to a solution of **20** (500 mg) in CH_2Cl_2 (25 mL). The mixture was stirred for 2 h at room temperature, washed with NH_4Cl aq. (sat) and extracted with CH_2Cl_2. The organic layer was washed with NaOH aq (0.5 M) and dried over anhydrous Na_2SO_4. The solvent was evaporated under vacuum and the crude product was purified by column chromatography (hexane/ethyl acetate 95:5) to give **21** in 84% yield. IR (film, cm^{-1}) ν_{max} 1193, 862; ^1H NMR ($CDCl_3$, 400 MHz): δ 1.33 (s, 3H, H-13), 1.26 (s, 3H, H-12), 1.58 (m, 2H, H-9), 2.4 (s, 3H, H-15), 1.81 (m, 1H, H-8), 2.81 (t, 1H, $J_{9,10a/b}$ = 6 Hz, H-10a), 2.76 (t, 1H, $J_{9,10a/b}$ = 6 Hz, H-10b), 3.46 (dq, 1H, $J_{7a/b,14}$ = 6.9, $J_{7a/b,8}$ = 7 Hz, H-7a), 3.40 (dq, 1H, $J_{7a/b,14}$ = 6.9, $J_{7a/b,8}$ = 7 Hz, H-7b), 5.15 (s, 3H, H-7′), 5.11 (s, 3H, H-7″a), 5.10 (s, 3H, H-7″b), 1.33 (d, 3H, $J_{7a/b,14}$ = 6.9 Hz, H-14), 6.89 (s, 1H, H-6), 6.91 (s, 1H, H-3), 7.41 (d, 2H, $J_{3',4';3'',4''}$ = 7.2 Hz, H4′, H4″), 7.48 (d, 4H, $J_{2',3';2'',3''}$ = 7.4 Hz, H2′, H-6′, H-2″, H-6″), 7.56 (dd, 4H, $J_{2',3';2'',3''}$ = 7.4, $J_{3',4';3'',4''}$ = 7.2 Hz, H-3′, H-5′, H-3″, H-5″); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 16.1 (C-15), 18.4 (C-13), 21.06 (C-14a), 21.09 (C-14b), 24.7 (C-12), 26.7 (C-9b), 27.1 (C-9a), 31.5 (C-7b), 31.9 (C-7a), 33.6 (C-8b), 33.9 (C-8a), 57.91 (C-11b), 57.92 (C-11a), 63.9 (C-10b), 64.3 (C-10a), 70.7 (C-7″b), 70.7 (C-7″a, C-7′), 111.4 (C-6), 115.3 (C-3), 127.60 (C-3″, C-5″), 127.65 (C-3′, C-5′), 128.0 (C-4′, C-4″), 128.78 (C-2″, C-6″), 128.81 (C-2′, C-6′), 133.3 (C-4b), 133.4 (C-4a), 137.5 (C-1a, C-1b), 138.15 (C-1′, C-1″), 150.01 (C-5b), 150.03 (C-5a), 151.0 (C-2b), 151.1 (C-2a); HRMS calcd for $C_{29}H_{34}O_3$ 430.5785, found 430.2516. Elemental analysis calcd for $C_{29}H_{34}O_3$: C, 80.89; H, 7.96; O, 11.15, found: C, 80.90; H, 8.82; O, 10.28.

5.1.2.7. 7*R,10*R**-10,11-Epoxy-10,11-dihydrocurcuhydro-quinone (2a) and 7*S**,10*R**-10,11-epoxy-10,11-dihydro-curcuhydroquinone (2b).** Pd/C (50%w) was added to a solution of **21** (50 mg) in dry *N*,*N*-dimethylformamide (DMF) and the mixture was placed under a H_2 atmosphere. The reaction mixture was stirred for 2 h and was then filtered through a silica column with EtOAc (25 mL) as the mobile phase. The crude product was washed with water (×5) to remove DMF. The organic layer was dried over anhydrous Na_2SO_4, filtered and the solvent was evaporated under vacuum. The mixture was purified by column chromatography (hexane/EtOAc 40%). Compounds **2a** and **2b** were obtained in 51% and 45% yield, respectively. The spectroscopic data have been reported previously for these compounds, with the same values for IR and HRMS, although the ^1H NMR and ^{13}C NMR spectra were obtained with deuterated acetone as solvent.

5.1.2.8. 7*R,10*R**-10,11-Epoxy-10,11-dihydrocurcuhydro-quinone (2a).** ^1H NMR (C_3D_6O, 400 MHz): δ 1.13 (s, 3H, H-13), 1.16 (d, 3H, $J_{7,14}$ = 7 Hz, H-14), 1.19 (s, 3H, H-12) 1.41 (q, 2H, $J_{8,9}$ = 7.9, $J_{9,10}$ = 7.6 Hz, H-9), 1.67 (td, 2H, $J_{7,8}$ = 7.1, $J_{8,9}$ = 7.9 Hz, H-8), 2.08 (s, 3H, H-15), 2.63 (t, 1H, $J_{9,10}$ = 7.6 Hz, H-10), 3.09 (tq, 1H, $J_{7,8}$ = 7.1, $J_{7,14}$ = 7.0 Hz, H-7), 6.56 (s, 1H, H-3), 6.60 (s, 1H, H-6); ^{13}C NMR (C_3D_6O, 100 MHz): δ 15.8 (C-15), 18.8 (C-13), 21.7 (C-14), 25.0 (C-12), 27.9 (C-9), 32.6 (C-7), 34.9 (C-8), 58.1 (C-11), 64.7 (C-10), 113.9 (C-3), 118.5 (C-6), 122.6 (C-4), 131.7 (C-1), 148.0 (C-5), 149.3 (C-2).

5.1.2.9. 7*S,10*R**-10,11-Epoxy-10,11-dihydrocurcuhydro-quinone (2b).** ^1H NMR (C_3D_6O, 400 MHz): δ 1.12 (s, 3H, H-13), 1.15 (d, 3H, $J_{7,14}$ = 6.8 Hz, H-14), 1.19 (s, 3H, H-12), 1.42 (ddd, 2H, $J_{8',9}$ = 6.3, $J_{9,10}$ = 6.3, $J_{8,9}$ = 13.4 Hz, H-9), 1.59 (ddt, 1H, $J_{7,8'}$ = 9.7, $J_{8,8'}$ = 12.8, $J_{8',9}$ = 6.3 Hz, H-8′), 1.75 (m, 1H, H-8), 2.07 (s, 3H, H-15), 2.61 (t,1H, $J_{9,10}$ = 6.3 Hz, H-10), 3.14 (dqd,1H, $J_{7,14}$ = 6.8, $J_{7,8}$ = 6.5, $J_{7,8'}$ = 9.7 Hz, H-7), 6.56 (s, 1H, H-3), 6.59 (s, 1H, H-6); ^{13}C NMR (C_3D_6O, 100 MHz): δ 15.8 (C-15), 18.8 (C-13), 21.5 (C-14), 25.0 (C-12), 27.7 (C-9), 32.3 (C-7), 34.4 (C-8), 57.9 (C-11), 64.2 (C-10), 113.9 (C-3), 118.2 (C-6), 122.5 (C-4), 131.7 (C-1), 148.0 (C-5), 149.2 (C-2).

5.1.3. Synthesis of compounds 3a and 3b to 14a and 14b

Each pair of diastereoisomers of series' C6, C5 and C4 (Fig. 2) was synthesized by the same methodology used to obtain compounds **2a** and **2b** described in the last section, although several changes were made to the conditions in certain stages. Reductions of the hydroxyl group in C-7 to obtain **4** and **10** and epoxidation of compounds in the C5 and C4 series' (**48**, **51**, **54**, **57**, **60**, **63** and **66**) were modified slightly. The selective cleavage of the benzyl groups was carried out under the same conditions for all compounds, but hydrogenation of **64** led to the reduced product **65** with the loss of the oxirane ring and only one hydroxyl group in C-10.

All synthetic details, including ^1H NMR ($CDCl_3$ or C_2D_6O, 400 MHz) and ^{13}C NMR ($CDCl_3$ or C_2D_6O, 100 MHz) data for these compounds are given in the Supplementary data.

5.2. Bioassay

5.2.1. General

For flow cytometry analysis, 10,000 live events were collected on a Cyan-ADP-MLE II flow cytometer (DakoCytomation™). Acquisition and analysis were performed using summit software (DakoCytomation™).

5.2.2. Cell culture and stimulation

The cell line Jurkat (American Type Culture Collection, Manassas, VA, USA) was maintained routinely in RPMI-1640 medium ((Roswell Park Memorial Institute medium) supplemented with 2 mM L-glutamine, 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 10% FBS (Fetal bovine serum), 1% nonessential amino acids (NEAA), 1% sodium pyruvate, and 1% penicillin/streptomycin at 37 °C).

5.2.3. CFSE cell division assay

For the CFSE cell division assay, cells were washed and resuspended in 0.5 mL PBS containing 5 μM CFDA-SE [5(and-6)-carboxyfluorescein diacetate succinimidyl ester] (Molecular Probes, Eugene, OR) and incubated at room temper-

ature for 5 min. Labelling was quenched by addition of an equal volume of FBS, cells were washed twice in PBS, resuspended in complete medium and allowed to proliferate for four days (cell number was determined by trypan blue exclusion). Data were collected on a flow cytometer and analyzed with appropriate software (DakoCytomation™ and ModFit LT™). Each compound was assayed in experiments coming at least from four different labeling. Values are expressed as Proliferation Index (PI) (represents the sum of cells in all generations divided by the calculated number of original parent cells present at the start of the experiment). PI is a measure of the increase in cell number in the culture, values lower than in control cultures represent inhibition of cellular division while values higher than in controls represent stimulation; Non-proliferative Fraction (NpF) (the number of cells in the parent generation at the time of data collection divided by the calculated number of cells present in the original culture). NpF represents the fraction in the original culture that has not proliferated during the course of the experiment. Values higher than the control indicate an increase in the non-proliferative effect, with the best results being NpF values close to 1. Parent (percentage of parent cells in the culture at the time of data collection) values higher than controls are indicative of an absence of cell division. Most populated generation (number of the most popular generation and the percentage of its cells at the moment of data collection), generation values lower than controls indicate inhibition of the cellular division.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.09.042.

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