4-Substituted-2-Methoxyphenol: Suitable Building Block to Prepare New Bioactive Natural-like Hydroxylated Biphenyls

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Abstract: A small collection of eugenol- and curcumin-analog hydroxylated biphenyls was prepared by straightforward methods starting from natural 4-substituted-2-methoxyphenols and their antitumoral activity was evaluated in vitro. Two curcumin-biphenyl derivatives showed interesting growth inhibitory activities on different malignant melanoma cell lines with IC_{50} ranging from 13 to 1 μM. Preliminary molecular modeling studies were carried out to evaluate conformations and dihedral angles suitable for antiproliferative activity in hydroxylated biphenyls bearing a side aliphatic chain.

Keywords: Curcumin, dihedral angle, hydroxylated biphenyls, malignant melanoma, synthesis.

1. INTRODUCTION

Hydroxylated biphenyl unit is embedded in many structures of bioactive natural products. Some of them are present in compounds of high biological relevance like ellagitannins, vancomicin, biphenomicins, others, structurally less sophisticated, are natural occurring dimers of 4-substituted-2-methoxy phenols [1, 2]. Generally, these dimers are 5,5'-disubstituted biphenyls produced by the C_2-symmetric coupling of the corresponding monomers present in softwood lignins [3].

Molecule having two symmetric potential binding moieties bearing a flexible unit of suitable length and nature would enhance binding affinity providing higher biological activity than that exerted by molecules lacking in these elements [4, 5].

Compared to 2-methoxy phenols, hydroxylated biphenyls manifest higher antioxidant activity and generally they are less toxic than the corresponding phenolic monomer [6, 7]. Dehydrodieugenol **2**, the natural C_2 symmetric dimer of eugenol **1**, manifests strong inhibitory effect on lipid peroxidation and scavenging ability for superoxide radicals (Fig. **1**) [8].

It is generally acknowledged that hydroxylated biphenyls are privileged molecules for proteins binding in comparison to other aromatic compounds in virtue of the flexible structure of the biphenyl that can be accommodated, with high level of specificity, in a wide variety of pockets present on protein surfaces [9].

In previous articles [10, 11] we reported that 6,6'-dibromo dehydrodieugenol **5** and 6,6'-dibromo dehydrodicreosol **6**, two C_2-symmetric hydroxylated biphenyls produced higher antiproliferative activity in different malignant melanoma cells (MM) compared to the corresponding conformationally flexible biphenyl no-bromo contained (Fig. **1**). Such activity resulted to be selective against tumor cells, without affecting human dermal fibroblast cells. No significant antitumoral activity was detected in the corresponding monomers.

Curcumin **7**, a pigment derived from the rhizome of *Curcuma longa,* is an effective compound in the treatment of a variety of cancers [12]. The chemical structure of curcumin **7** consists of two 2-methoxy phenol rings, each of them coplanarly linked to an α,β-unsaturated β-diketone moiety. Unfortunately, the low plasma solubility of curcumin **7** and the easy production of metabolites under oxidative or reductive bio-condition restricts the development of this natural occurring compound in therapy programs [13]. Nevertheless, a wide spectrum of biological and pharmacological activities of curcumin **7** makes this compound a promising pharmacological lead [14, 15]. Recently, we have prepared and tested *in vitro* and *in vivo* biphenyl **8** (Fig. **1**), a curcumin-analog featured with two α,β-unsaturated ketone chains at 5,5'-positions of the aromatic rings [16]. Biphenyl **8** was revealed to be more effective in inhibiting malignant melanoma and neuroblastoma cells when compared to curcumin **7** [16].

Taking together all data achieved on hydroxylated biphenyls, we considered that 4-substituted-2-methoxy phenols would appear interesting starting materials to prepare new C_2 symmetric hydroxylated biphenyls sharing the feature of two identical aliphatic chains at the 5,5′ positions differing in functionalities and length. An antiproliferative activity against MM cells proliferation would be expected.

2. MATERIALS AND METHODS

2.1. General

Melting points are uncorrected. All ^1H NMR and ^{13}C NMR spectra were recorded in $CDCl_3$ solution at 399.93 MHz and 100.57 MHz, respectively. Chemical shifts are given in ppm (δ); multiplicities are indicated by s (singlet), d

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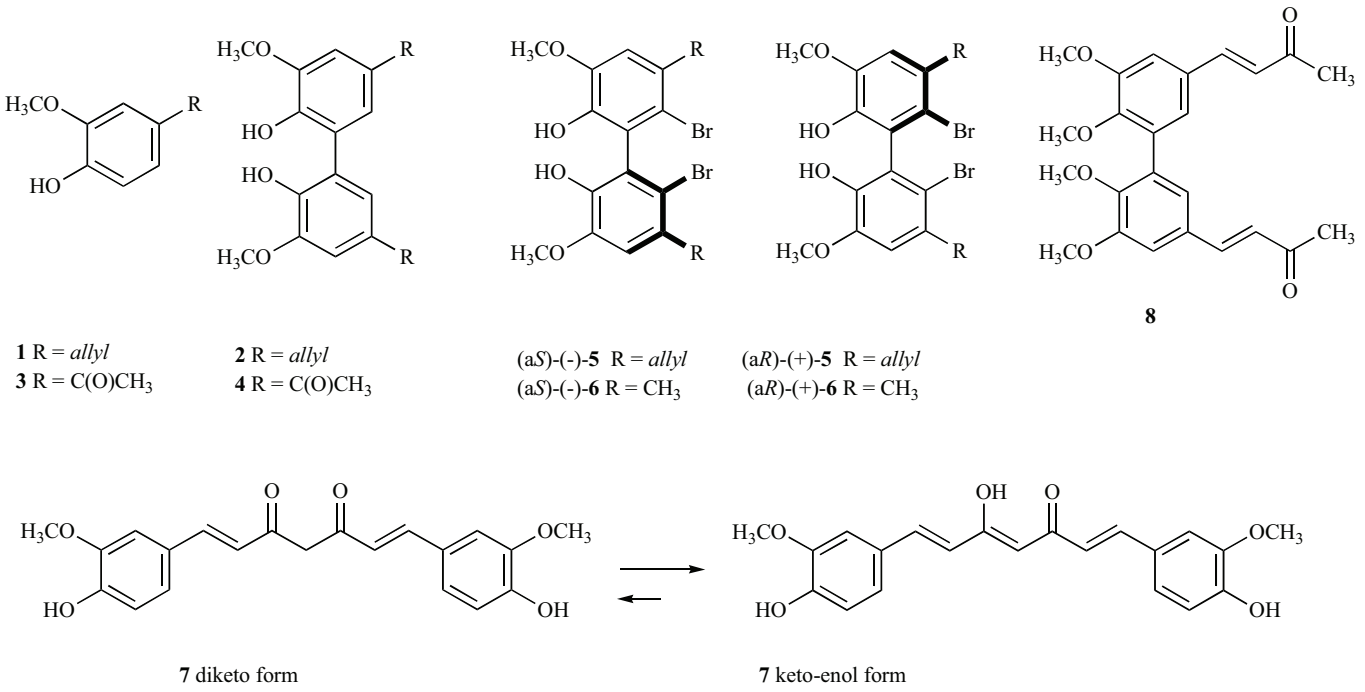


Fig. (1). 4-Substituted-2-methoxyphenols and dimers.

(doublet), t (triplet), ddt (doublet doublet triplet), m (multiplet) or bs (broad singlet). Elemental analyses were performed using an elemental analyser Perkin-Elmer model 240 C. Optical rotations were measured with a Perkin-Elmer 343 spectropolarimeter. Toluene was freshly distilled from sodium benzophenone ketyl and acetone was dried over $CaCl_2$ and distilled before use. Ethanol (EtOH) grade 96% was used. All reagents were of commercial quality and used as purchased. Flash chromatography was carried out with silica gel 60 (230-400 mesh, Kiesgel, EM Reagents) eluting with appropriate solution in the stated v:v proportions. The purity of all new compounds was judged to be >98% by ^{1}H-NMR and ^{13}C-NMR spectral determination. Biphenyls **15** and **16** were prepared as previously described [17, 18].

2.2. Chemistry

General Procedure for the Synthesis of Compounds 9 and 13.

To a solution of biphenyl (1 eq) and K_2CO_3 (1.1 eq) in dry acetone (15 mL) CH_3I (1.1 eq) was added dropwise, at rt under N_2. The solution was stirred at 50 °C for 12 h, water was added and the organic phase extracted with ether. The crude, dried over Na_2SO_4, gave the corresponding 2,2'-dimethoxy biphenyl derivative that was purified by flash chromatography using CH_2Cl_2 as an eluent.

2,2',3,3'-Tetramethoxy-5,5'-di(2-propenyl)–6,6'-dibromo-1,1'-biphenyl (9)

Pale yellow solid (0.95 g, 90%): mp 113-4 °C; ^{1}H NMR δ 3.54 (m, 4H), 3.67 (s, 6H), 3.88 (s, 6H), 5.04-5.15 (series of m, 4H), 5.91 (ddt, J = 20.1, 13.6, 8.8 Hz, 2H), 6.86 (s, Ar, 2H); ^{13}C NMR δ 41.04, 56.04, 60.68, 113.77, 116.69, 116.85, 134.80, 135.26, 136.03, 145.87, 152.09; Anal. Calcd for $C_{22}H_{24}Br_2O_4$: C, 51.76; H, 4.70; Found: C, 51.90; H, 4.68.

2,2',3,3'-Tetramethoxy-5,5'-dipropyl-1,1'-biphenyl (13)

Colourless oil (1.49 g, 92%); ^{1}H NMR δ 0.94 (t, J = 7.2 Hz, 6H), 1.65 (m, 4H), 2.50 (t, J = 7.6 Hz, 4H), 3.62 (s, 6H), 3.87 (s, 6H), 6.69 (d, J = 1.6 Hz, Ar, 2H), 6.73 (d, J = 1.6 Hz, Ar, 2H); ^{13}C NMR δ 13.82, 24.52, 37.87, 55.75, 60.56, 111.71, 123.03, 132.42, 137.68, 144.66, 152.30; Anal. Calcd for $C_{22}H_{30}O_4$: C, 73.74; H, 8.38; Found: C, 73.89; H, 8.36.

General Procedure for the Synthesis of Compounds 10, 11 and 12.

A solution of 5,5'-diallyl biphenyl derivative (1 eq) and 10% Pd/C (10% w/w) in EtOH (10 mL) was stirred at rt under 1-2 atm. of H_2 for 2 h. The solution was filtered through a pad of celite and washed with EtOH to obtain a pure 5,5'-dipropyl biphenyl derivative.

2,2'-Dihydroxy-3,3'-dimethoxy-5,5'-dipropyl-1,1'-biphenyl (10)

Colourless solid (1.92 g, 95%): mp 147-8 °C; ^{1}H NMR δ 0.95 (t, J = 7.2 Hz, 6H), 1.65 (m, 4H), 2.56 (t, J = 7.6 Hz, 4H), 3.95 (s, 6H), 6.03 (bs, 2H), 6.72 (s, Ar, 2H), 6.75 (s, Ar, 2H); ^{13}C NMR δ 13.89, 24.77, 37.87, 56.07, 110.01, 110.64, 122.97, 124.49, 134.68, 140.49; Anal. Calcd for $C_{20}H_{26}O_4$: C, 72.73; H, 7.88; Found: C, 72.81; H, 7.90.

2,2'-Dihydroxy-3,3'-dimethoxy-5,5'-dipropyl-6,6'-dibromo-1,1'-biphenyl (11)

Colourless solid (0.86 g, 85%): mp 135-7 °C; ^{1}H NMR δ 1.01 (t, J = 7.2 Hz, 6H), 1.68 (m, 4H), 2.72 (t, J = 7.4 Hz,

4H), 3.91 (s, 6H), 5.52 (bs, 2H), 6.1 (s, Ar, 2H); ^{13}C NMR δ 13.88, 23.49, 38.72, 55.97, 103.67, 11.83, 118.71, 133.51, 134.26, 164.36; Anal. Calcd for $C_{20}H_{24}Br_2O_4$: C, 49.38; H, 4.94; Found: C, 49.45; H, 4.96.

2,2',3,3'-Tetramethoxy-5,5'-dipropyl-6,6'-dibromo-1,1'-biphenyl (12)

Light brown solid after flash chromatography using CH_2Cl_2 as eluent (1.63 g, 81%): mp 89-90°C; ^{1}H NMR δ 0.99 (t, *J* = 7.2 Hz, 6H), 1.66 (m, 4H), 2.74 (m, 4H), 3.66 (s, 6H), 3.88 (s, 6H), 6.48 (s, Ar, 2H); ^{13}C NMR δ 13.87, 23.43, 38.86, 55.75, 60.37, 113.28, 116.50, 134.56, 137.70, 145.21, 151.64; Anal. Calcd for $C_{22}H_{28}Br_2O_4$: C, 51.36; H, 5.45; Found: C, 51.50; H, 5.44.

Homochiral and meso 2,2'-dihydroxy-3,3'-dimethoxy-5,5'-di(2-bromo-propyl)-1,1'-biphenyl (14)

To a solution of **2** (2 g, 6.13 mmol) in acetic acid (10 mL), HBr (5 mL, 40% in acetic acid) was added in one pot. The reaction mixture was stirred at rt for 5 h. Water (150 mL) was added to the mixture and the resulting precipitate was filtered, dissolved in CH_2Cl_2 and dried to obtain **14** as a 75:25 mixture of diastereoisomers. The brown solid was purified by flash chromatography using a 1:2 mixture of AcOEt: petroleum ether, as eluent, (2.68 g, 90%). Diastereomer (*homochiral*): mp 130-2 °C; ^{1}H NMR δ 1.72 (d, *J* = 8.8 Hz, 6H), 2.80 (AB system, *J* = 9.6, 18.4 Hz, 4H), 3.95 (s, 6H), 4.30 (m, 2H), 6.10 (bs, 2H), 6.75 (d, *J* = 2 Hz, Ar, 2H), 6.77 (d, *J* = 2 Hz, Ar, 2H); ^{13}C NMR δ 25.69, 47.32, 50.98, 56.20, 111.35, 124.04, 124.18, 130.45, 141.57, 147.18; Anal. Calcd for $C_{20}H_{24}Br_2O_4$: C, 49.38; H, 4.94; Found: C, 49.51; H, 4.96. Diastereomer (*meso* form): mp 101-2 °C; ^{1}H NMR δ 1.69 (d, *J* = 6.6 Hz, 3H), 1.73 (d, *J* = 6.6 Hz, 3H), 3.01 (AB system, *J* = 7.2, 14 Hz, 2H) 3.15 (AB system, *J* = 6.8, 14 Hz, 2H), 3.85 (s, 3H), 3.91 (s, 3H), 4.24-4.34 (series of m, 2H), 5.23 (bs, 1H), 5.62 (bs, 1H), 6.61 (d, *J* = 2 Hz, Ar, 1H), 6.71 (d, *J* = 2 Hz, Ar, 1H), 6.82 (m, Ar, 2H); ^{13}C NMR δ 25.68, 25.75, 47.10, 47.47, 50.16, 50.99, 56.12, 56.14, 111.31, 112.62, 123.04, 123.44, 123.80, 124.19, 129.62, 131.56, 136.56, 141.93, 146.60, 151.37; Anal. Calcd for $C_{20}H_{24}Br_2O_4$: C, 49.38; H, 4.94; Found: C, 49.56; H, 4.95.

2,2'-Dibenzyloxy-3,3'-dimethoxy-5,5'-diacetyl-1,1'-biphenyl (17)

To a solution of diapocynin **4** (2.0 g, 6.0 mmol) in dry acetone (50 ml), K_2CO_3 (1.8 g, 13.2 mmol) was added under nitrogen. The mixture was heated at 50 °C for 1 h, then $PhCH_2Br$ (2.3 g, 13.2 mmol) was added and the mixture was heated again at 50 °C for 12 h. After cooling, the reaction mixture was treated with 10% HCl and the organic phase was extracted with CH_2Cl_2. After flash chromatography (petrolium ether: AcOEt) compound **17** (2.9 g, 94%) was achieved as a white solid: mp 102-103 °C (lit. 101 °C) [19]; ^{1}H NMR δ 2.46 (s, 6H), 3.98 (s, 6H), 4.88 (s, 4H), 6.96-7.01 (series of m, Ar, 4H), 7.13-7.22 (series of m, Ar, 6H), 7.39 (d, *J* = 2.4 Hz, Ar, 2H), 7.62 (d, *J* = 2.4 Hz, Ar, 2H); ^{13}C NMR 26.62, 55.34, 74.85, 110.95, 125.49, 128.07, 128.31, 128.33, 132.09, 132.81, 137.28 150.11, 153.27, 197.37; Anal. Calcd for $C_{32}H_{30}O_6$: C, 75.28; H, 5.92; Found: C, 75.22; H, 4.94.

3-[5,6,2',3'-Tetramethoxy-5'-(2-methoxycarbonyl-vinyl)-biphenyl-3-yl]-acrylic Acid Methyl Ester (18)

To a solution of **15** (1 g, 2.8 mmol) in MeOH (12 mL) and CH_2Cl_2 (224 mL), DDQ (4.4 g, 19.6 mmol) was added and the resulting mixture was stirred at rt for 5 h. The crude product was washed with brine. Solids were filtered and the organic layer was extracted with CH_2Cl_2, dried, and produced to effort a yellow solid. Flash chromatography under neutral Al_2O_3 (petroleum ether: AcOEt) (0.50 g 40%): mp 151-152 °C; ^{1}H NMR δ 3.67 (s, 6H); 3.78 (s, 6H), 3.93 (s, 6H), 6.34 (d, *J* = 16.0 Hz, 2H), 7.01 (d, *J* = 2.4 Hz, Ar, 2H), 7.08 (d, *J* = 2.4 Hz, Ar, 2H), 7.61 (d, *J* = 16 Hz, 2H); ^{13}C NMR δ 51.91, 56.14, 61.08, 111.04, 117.26, 123.97, 130.04, 132.57, 144.67, 149.09, 153.18, 167.67; Anal. Calcd for $C_{24}H_{26}O_8$: C, 65.15; H, 5.29; Found: C, 65.21; H, 5.27.

Enantiopure-3-{5'-[2-(2-Hydroxy-1-methyl-propoxycarbonyl)-vinyl]-5,6,2',3'-tetramethoxy-biphenyl-3-yl}-acrylic acid 2-hydroxy-1-methyl-propyl Ester (19)

To a solution of **15** (0.5 g, 1.4 mmol) in CH_2Cl_2 (50 ml), enantiopure (2*R*,3*R*)-(-)-2,3 butandiol (0.25 g 2.8 mmol) and DDQ (2.3 g, 10 mmol) were added and the mixture was stirred at rt for 5 h, then quenched with brine. The organic layer was extracted with CH_2Cl_2 and then dried. Flash chromatography under neutral Al_2O_3 using a 2:1 mixture of AcOEt: petroleum ether, as an eluent, gave **19** as a brown solid (0.23 g, 30%): mp 86-88 °C; ^{1}H NMR δ 1.21 (d, *J* = 6.4 Hz, 6H), 1.27 (d, *J* = 6.4 Hz, 6H), 3.68 (s, 6H), 3.81 (q, *J* = 5.6 Hz, 2H), 3.93 (s, 6H), 4.87 (q, *J* = 5.6 Hz, 2H); 6.36 (d, *J* = 16.0 Hz, 2H), 7.04 (d, *J* = 2.0 Hz, Ar, 2H), 7.09 (d, *J* = 2.0 Hz, Ar, 2H), 7.62 (d, *J* = 16.0 Hz, 2H); ^{13}C NMR δ 19.30, 21.1, 56.1, 61.0, 70.4, 75.1, 111.1, 117.5, 124.0, 129.9, 132,5, 144.9, 149.1, 153.1, 166.9; Anal. Calcd for $C_{30}H_{38}O_{10}$: C, 64,50; H, 6,86; Found: C, 64.61; H, 6.65; $[\alpha]^{20}_D$ = -15.2 (c = 0.4, CH_2Cl_2); $[\alpha]^{20}_{546}$ = -20.6 (c = 0.4, CH_2Cl_2).

General Procedure for the Synthesis of Compounds 22 and 23

Diester (1 eq) was refluxed for 12h with a solution of KOH (1.9 eq) in EtOH (50 mL). Then, the mixture was acidified with HCl 10%. The resulting precipitate was filtered and washed with EtOH (50 mL). The liquid layers were evaporated to provide the corresponding diacid without purification.

3-[5'-(2-Carboxy-vinyl)-5,6,2',3'-tetramethoxy-biphenyl-3-yl]-acrylic Acid (22)

Beige solid (0.35 g, 85%): mp 269-270 °C; ^{1}H NMR δ 3.71 (s, 6H), 3.95 (s, 6H), 6.35 (d, *J* = 16.0 Hz, 2H), 7.04 (s, Ar, 2H), 7.13 (s, Ar, 2H), 7.73 (d, *J* = 16.0 Hz, 2H); ^{13}C NMR δ 56.16, 61.12, 111.17, 116.61, 122.41, 129.76, 132.58, 146.74, 149.46, 153.21, 171.62; Anal. Calcd for $C_{24}H_{22}O_8$: C, 63,76; H, 5,35; Found: C, 63.59; H, 5.33.

4-[5'-(3-Carboxy-3-hydroxy-acryloyl)-5,6,2',3'-tetramethoxy-biphenyl-3-yl]-2-hydroxy-4-oxo-but-2-enoic Acid (23)

Beige solid (0.6 g, 78%): mp 242-243 °C; ^{1}H NMR δ (DMSO d_6): 3.65 (s, 6H,), 3.95 (s, 6H), 7.14 (s, 2H), 7.60 (d, *J* = 1.6 Hz, Ar, 2H), 7.67 (d, *J* = 1.6 Hz, Ar, 2H); ^{13}C NMR ($CDCl_3$) δ 56.7, 61.1, 98.7, 112.0, 124.1, 130.7, 132.2, 151.8, 153.1, 163.9, 168.5, 191.2; Anal. Calcd for $C_{24}H_{22}O_{12}$: C, 57,37; H, 4,41; Found: C, 57.42; H, 4.43.

General Procedure for the Synthesis of Compounds 20 and 21

To a solution of diketone (1 eq) in dry toluene (20 mL), NaH (2 eq, 60% in mineral oil) was added at rt under N_2. After 10 min, diethyl oxalate (1 eq) was added dropwise and the mixture let to stir under reflux for 5 h. The mixture was cooled at room temperature, acidified with HCl 10% and extracted with CH_2Cl_2. The organic phase, dried, provided a residue which was washed with Et_2O and filtered to yield the β-diketo ester.

4-[5'-(2-Ethoxycarbonyl-3-hydroxy-acryloyl)-5,6,2',3'-tetramethoxy-biphenyl-3-yl]-2-hydroxy-4-oxo-but-2-enoic acid ethyl ester (20)

Orange solid (0.4 g, 93%): mp 187-188 °C; ^{1}H NMR δ 1.39 (t, *J* = 7.2 Hz, 6H), 3.78 (s, 6H), 4.00 (s, 6H), 4.37 (q, *J* = 7.2 Hz, 4H), 7.02 (s, 2H), 7.51 (d, *J* = 2.4 Hz, Ar, 2H), 7.64 (d, *J* = 2.4 Hz, Ar, 2H); ^{13}C NMR δ 14.3, 56.3, 61.2, 62.8, 98.2, 111.4, 124.0, 130.6, 131.9, 152.2, 153.2, 162.5, 168.5, 190.7; Anal. Calcd for $C_{28}H_{30}O_{12}$: C, 60,21; H, 5,41; Found: C, 60.30; H, 5.40.

1-[6,2'-Bis-benzyloxy-5'-(3-hydroxy-4-oxo-hex-2-enoyl)-5,3'-dimethoxy-biphenyl-3-yl]-3-hydroxy-hex-2-ene-1,4-dione (21)

Orange oil (1.14 g, 82%): ^{1}H NMR δ 1.39 (t, *J* = 7.2 Hz, 6H), 4.00 (s, 6H), 4.38 (q, *J* = 7.2 Hz, 4H), 4.95 (s, 4H), 6.92(s, 2H), 6.98 (d, *J* = 7.2 Hz, Ar, 4H), 7.10-7.19 (series of m, Ar, 6H), 7.28 (d, *J* = 1.8 Hz, Ar, 2H), 7.63 (s, *J* = 1.8 Hz, Ar, 2H); ^{13}C NMR δ 14.1, 56.19, 62.61, 74.69, 98.20, 111.04, 123.99, 127.92, 128.07, 128.14, 130.33, 132.46, 136.81, 150.65 153.12, 162.31, 168.07, 190.49; Anal. Calcd for $C_{40}H_{38}O_{10}$: C, 70,78; H, 5,64; Found: C, 70.84; H, 5.66.

2.3. Molecular Modeling

Model compounds **8-14** and **17**, **18**, **20**, **21**, **23** were constructed with standard bond lengths and angles from the fragment database with MacroModel 6.0 [20, 21] using a Silicon Graphics O2 workstation running on IRIX 6.3. Minimization of structures was performed with the MacroModel/BachMin 6.0 [20, 21] program using the AMBER force field.

An extensive conformational search was carried out using the Monte Carlo/Energy minimization method for all the compounds considered in the study (energy difference between the generated conformation and the current minimum set to 5.0 Kcal/mol). Minimization of structures was performed with Sybyl 6.3 [22], method BFGS (Davidon-Fletcher-Powell), max interations 10000, energy setup force field Tripos, Representative minimum energy conformations of these compounds were optimized using the quantum chemistry program Gaussian 03W [23] with Density Functional Method B3LYP and 6-311G basis set. The visualization of the results obtained was performed by Gaussian View 4.1 [24].

2.4. Biological Assay

Cell Lines

Malignant Melanoma cell lines (WM266, CN, LB24Dagi, PNP) were kindly provided by Drs. D. Castiglia and S. D'Atri at the "Instituto Dermopatico dell'Immacolata" in Rome. They were established as primary short-term cell cultures from tumor samples of donors patients with documented diagnosis of malignant melanoma after obtaining their informed consent, as previously described [25]. Cells were cultured to confluence in tissue culture flasks using either Dulbecco's minimal essential medium (DMEM) or RPMI medium (Invitrogen, Carlsbad, CA) supplemented with 10% FBS and penicillin/streptomycin [100 IU (50 μg)/ml] in a humidified 5% CO_2 atmosphere at 37°C.

Cell Proliferation Assays

Cells were plated in a 96-well plates (3×10^3/well) in complete medium. After 24 hours, medium was removed and replaced on days 1, 3 and 5 by fresh medium containing or not (control) various doses of **9**-**14** or **17**, **18**, **20**, **21**, **23** as described in the legend of Fig. (**2**). Each experiment was performed in quadruplicate. Cells were observed with inverted microscope after every 24 hours to check morphological changes, and suffering or cell death. The percentage of cell proliferation was estimated on day 6 (96 hrs treatment) by a known colorimetric assay [26] modified as follows: cells were fixed for 20 min at a rt with 4% paraformaldehyde (PFA), stained with 0.1% crystal violet in 20% methanol for 20 min, washed with PBS, solubilized with 10% acetic acid and were read at 595 nm in a microplate reader (SpectraFluor Plus, Tecan, Austria).

3. RESULTS AND DISCUSSION

3.1. Synthesis

Based on our previous results in hydroxylated biphenyls with antiproliferative activity [17,18] we prepared a small collection of eugenol- and curcumin-biphenyl derivatives starting from eugenol **1** and apocynin **3**, respectively (Scheme **1**). According to known procedures, dehydrodieugenol **2** and diapocynin **4** were prepared starting from eugenol **1** and apocynin **3**, respectively. Although both monomers belong to the family of 2-methoxy phenols, substituents in para to phenol-OH group require different oxidative coupling conditions and reagents.

Eugenol **1** was treated with a solution of NH_4OH and $K_3Fe(CN)_6$ in acetone-water at room temperature in open air

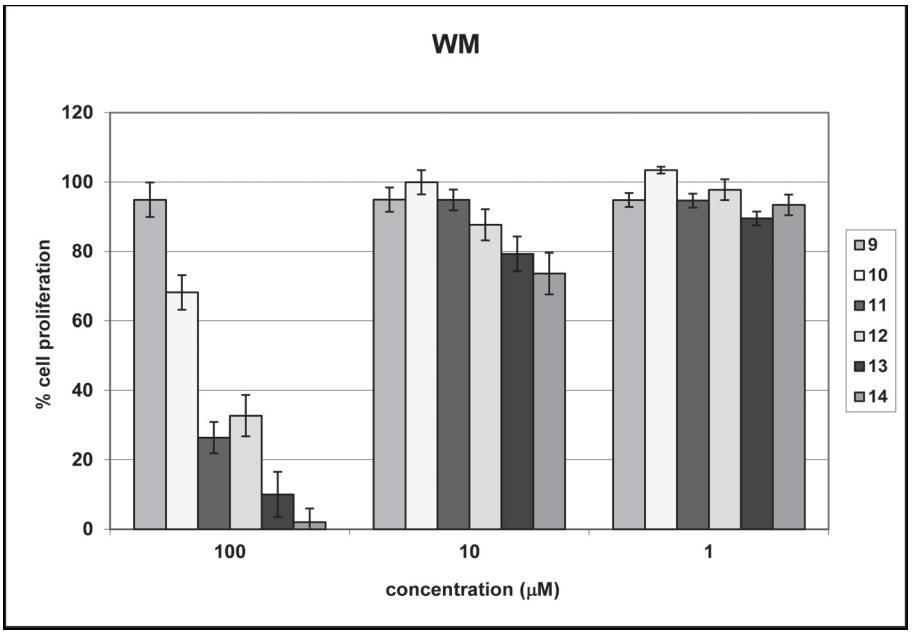
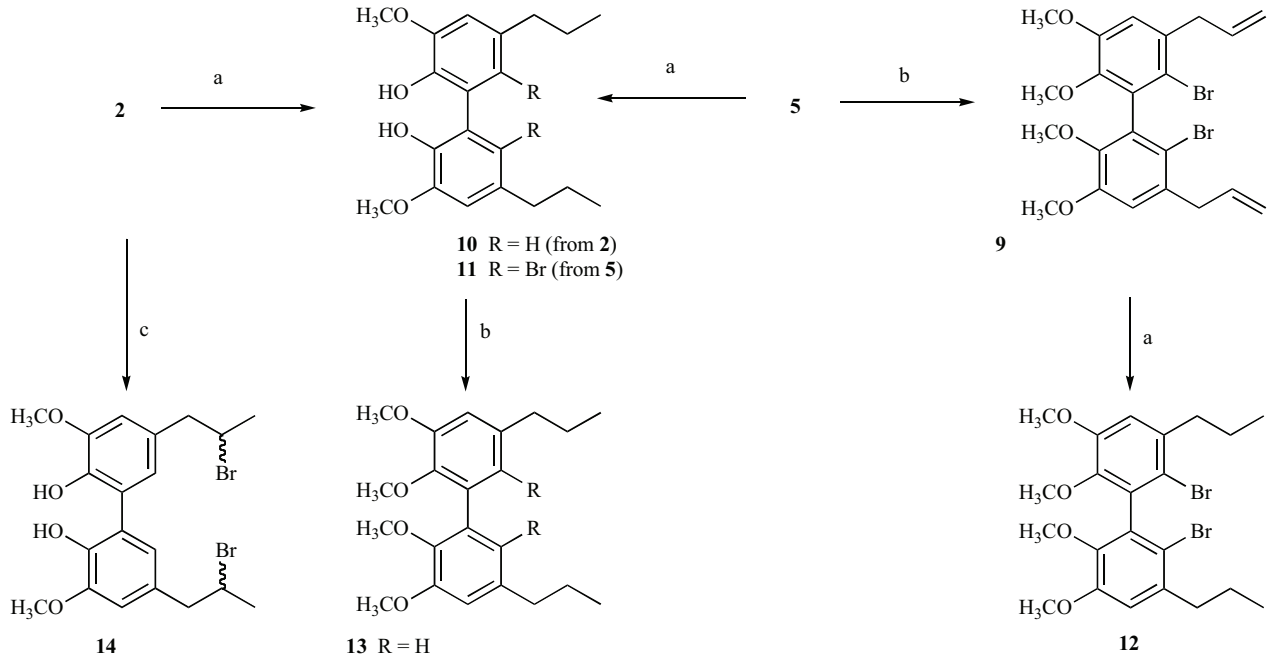


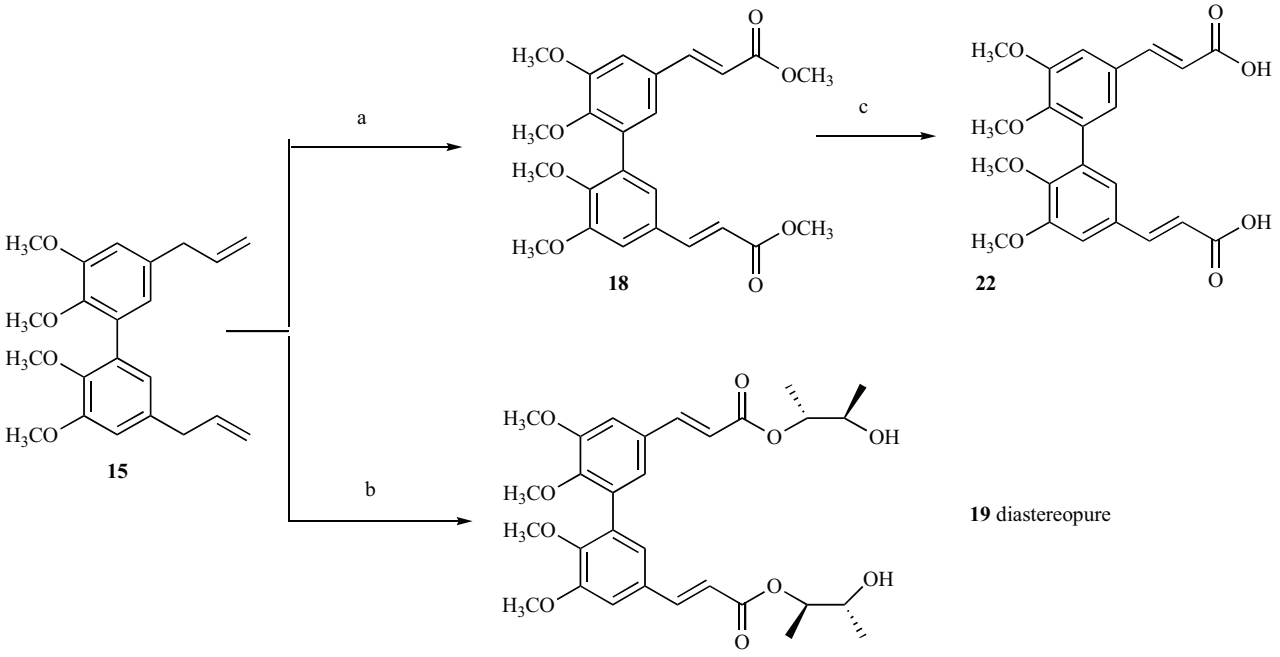
Fig. (2). Effect of the eugenol-analogue biphenyl compounds on the growth of human malignant melanoma cell lines. Data from a single MM cell line (WM) is reported as exemplificative of all. Cells were cultured in the presence of 1, 10, 100 μM of each compound (**9-14**) for 6 days. Cell proliferation was estimated and results are expressed as percentage of cell growth representing the average (± SD) of quadruplicate cultures performed twice.



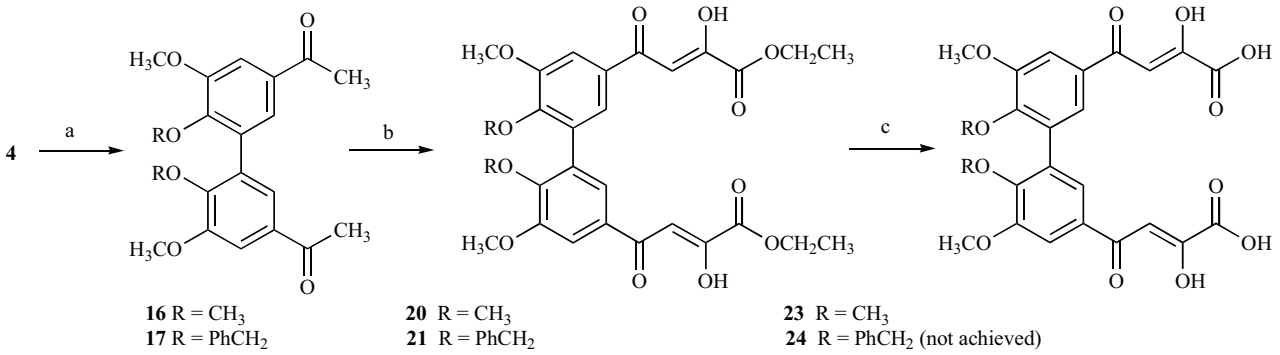
Scheme (1). Synthesis of eugenol-biphenyl derivatives **9-14**. Reagents and Conditions: (a) H_2, 10% Pd/C, EtOH, rt, 2h; (b) K_2CO_3, CH_3I, acetone, 50 °C, 12h; (c) HBr, AcOH, rt, 5h.

[27]. Dehydrodieugenol **2** was obtained as a colorless solid in 95% yield after recrystallization from absolute ethanol. Dehydrodieugenol **2** was further brominated in the presence of Br_2 and then treated with Zn dust in Et_2O to give dimer **5** in overall 90% yield [10]. Commercial apocynin **3** was treated with a stoichiometric excess of $K_2S_2O_8/FeSO_4$ at room temperature in open air in a mixture of water/acetone to give diapocynin **4** in 80% yield [28].

Treatment of dehydrodieugenol **2**, 6,6'-dibromo derivatives **5** and **9** in the presence of H_2 and catalytic amount of 10% Pd/C in absolute EtOH at rt, gave complete reduction of the two allyl in *n*-propyl groups. Biphenyls **10**, **11** and **12** were achieved in high yields as solids. Biphenyls **9** and **13** were obtained in high yields by the protection of phenol-OH groups of **5** and **10** in the presence of CH_3I and K_2CO_3 in dry acetone, respectively.



Scheme (2). Synthesis of curcumin-biphenyl derivatives **18**, **19** and **22**. Reagents and Conditions: (**a**) DDQ, MeOH, CH_2Cl_2 rt, 5h; (**b**) DDQ, (2*R*, 3*R*)-(-)-2,3-butandiol, CH_2Cl_2 rt, 5h; (**c**) KOH, EtOH, reflux, 12h.



Scheme (3). Synthesis of curcumin-biphenyl derivatives **17**, **20**, **21**, **23**, **24**. Reagents and Conditions: (a) K_2CO_3, $PhCH_2Br$, acetone, 50 °C, 12h; (b) NaOH, $(CO_2Et)_2$, toluene, rt, 5h; (c) KOH, EtOH, reflux, 12h.

According to Marknovnikov's rule, dibromoderivative **14,** obtained by the bromination of **2,** was achieved as a mixture of *meso* and *homochiral* diastereomers in 25:75 ratio, respectively (Scheme **1**). We were able to separate and characterize both couples of diastereomers by flash chromatography.

In solution, curcumin **7** exists mainly in the *syn* keto-enol tautomeric form because of the strong intramolecular hydrogen bond and high double bond conjugation [29].

It is generally acknowledged that the β-diketoacid moiety enolizes at the α position to form the resultant stable *Z* enol tautomer. The presence of a carbonyl function in conjugation with the enolic double bond allows the enol to be the predominant form. Although less investigated, also β-diketoesters are pretty stable in the enol tautomeric form [30].

We hypothesized that the presence of a biphenyl scaffold in a curcumin-analog structure would control rigidity at the two aromatic moieties and thus, it might play an important role to enhance antitumoral activity. According to our assumption, we prepared C_2 symmetric hydroxylated biphenyls incorporating different and stable *Z* enol tautomers and α,β-unsaturated groups at the 5,5'-positions starting from dehydrodieugenol **2** and diapocynin **4**. Since it was reported that protection of the phenol-OH groups of curcumin through methylation improved its stability, hydroxyl groups of biphenyls **2** and **4** were protected with methyl or benzyl group to give biphenyls **15** [17], **16** [18] and **17** (Schemes **2** and **3**).

When dimethyl-dehydrodieugenol **15** was treated with 3.6 eq of 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ) in CH_2Cl_2 at rt and quenched with excess of alcohol [31], α,β-unsaturated esters **18** and **19** were achieved. (Scheme **2**). Quantitative *Claisen* condensation of biphenyls **16** and **17** in the presence of diethyl oxalate and a base was carried out to give β-diketo esters **20** and **21**, respectively (Scheme **3**).

Alkaline hydrolysis of biphenyls **18** and **20** in refluxing ethanol gave α,β-unsaturated acid **22** and β-diketo acid **23**,

Table 1. IC_{50} values (μM) of curcumin 7 and biphenyls 8, 17, 18, 20, 21 and 23.

Compounds	*In vitro* IC_{50} (μM)			
	CN	WM	LB	PNP
7	n.d.	11.5	9.8	8.6
8	1.8	1.0	1.2	1.2
17	> 100	> 100	> 100	> 100
18	38.0	58.0	> 100	80.0
20	11.0	15.0	50.0	61.0
21	13.0	8.2	11.2	n.d.
23	47.0	51.0	> 100	> 100

respectively (Scheme **2** and **3**). In solution, complete keto-enol form was observed by NMR spectroscopy for diesters **20**, **21** and diacid **23**. Several attempts to achieve β-diketo acid **24** from compound **21** by alkaline hydrolysis, failed. The reaction gave a mixture of compounds in which ketone **17** was isolated in 58% yield.

Oxidation of **15** with DDQ in the presence of MeOH or diol gave α,β-unsaturated esters **18** and **19** in high yields with complete *E*-stereoselectivity at the two double bonds. This synthetic strategy appeared to be more straightforward, costless and stereoselective at the double bond compared to the classical *Wittig* reaction generally applied to prepare α,β-unsaturated aryl esters. No intramolecular adduct was observed after the reaction of **15** with (2*R*, 3*R*)-(-)-2,3-butandiol, even when the diol was used in equimolar ratio. An intermolecular adduct was detected in such a small quantity that purification from the crude of reaction was not carried out.

3.2. Biological Activity

In vitro cell proliferation assays of the new biphenyls were carried out by using tumour cell lines as described in Material and Methods. In a first assay, activity of eugenol-biphenyl derivatives **9-14** was evaluated. Diastereomer **14** was tested only in *homochiral* form. Three different MM cell lines were cultured in the presence of different concentrations (1, 10, 100 μM) of each biphenyl compound to be tested (Fig. **2**).

Data obtained from one of the MM cell lines (WM) treated with each of the biphenyls **9-14** are reported in Fig. (**2**) and they reflect the behaviour of all the cell lines tested. Compounds **9** and **10** were formed to be less effective on cell growth inhibition even at the highest concentration tested. Instead, compounds **11** and **12** showed a better antiproliferative activity (respectively 75% and 65% of growth inhibition) at the same high concentration (100 μM) while at the lower concentration of 10 μM only the compounds **13** and **14** were able to inhibit MM cell proliferation of about 30% and 20% respectively.

Subsequently, curcumin-biphenyl derivatives **17**, **18**, **20**, **21** and **23** were tested for their capability to inhibit cell growth on cultured MM cells by *in vitro* assays. Four different MM cell lines were grown up to 6 days in the presence of increasing concentrations (1 to 100 μM) of each biphenyl compound to be tested (Table **1**).

Relative 50% cell growth inhibition concentration (IC_{50}) values were estimated for each compound and they are summarized in Table **2**. Compound **17** did not show any significant antiproliferative activity up to the highest dose used in our experiments. Compounds **18** and **23** show a variable efficacy depending on the MM cell line. Only compound **21** showed a good antitumoral efficacy on all the cell lines examined with IC_{50} comparable with that of curcumin **7**. IC_{50} values of biphenyl **8**, previously prepared and tested by us on the same cell lines [16], were added in Table **1**.

Comparing antiproliferative activity data between eugenol- and curcumin-biphenyl derivatives, we evidenced that the curcumin-biphenyl derivatives are more promising leading molecules to be developed as antitumoral agents against MM cells.

3.3. Evaluation of Dihedral Angle

In order to gain insights on conformations of the new biphenyls, dihedral angle of low energy conformers of compounds **9-14** and **17**, **18**, **20**, **21** and **23** were evaluated. The dihedral angle Φ between the two benzene rings which depends on the size, number and the position of the substitu-

Table 2. Dihedral angle of biphenyls 9-14.

Compounds	Dihedral Angle
9	88.2(2)°
10	139.0(2)°
11	89.0(7)°
12	89.6(5)°
13	49.8(6)°
14[a]	39.8(2)°

[a] homochiral

Table 3. Dihedral angle of biphenyls 8, 17, 18, 20, 21 and 23.

Compounds	Dihedral Angle
8	45.1(5)°
17[a]	57.3(9)°
18	124.7(0)°
20	121.8(5)°
21	57.1(0)°
23[a]	86.9(1)°

[a] Φ 55.8° by crystallographic analysis [19].

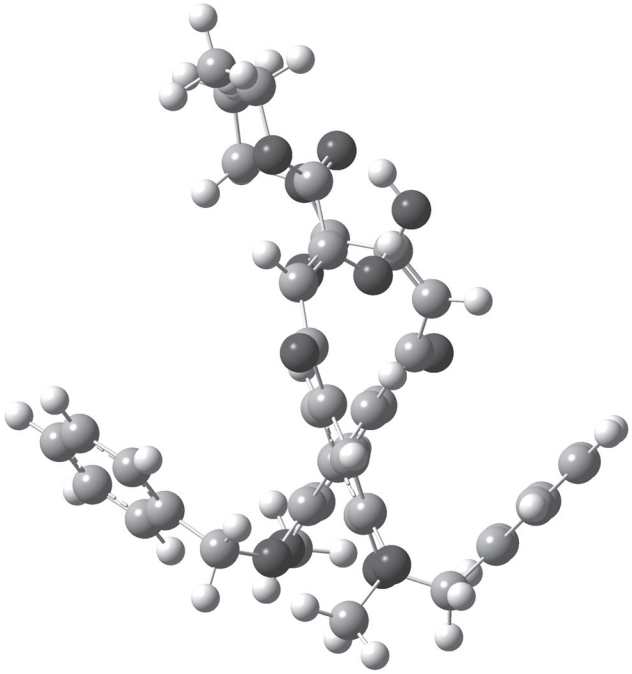


Fig. (3). Molecular structure of low-energy conformer **21**. Oxygen atoms are displayed with dark grey, carbon atoms with grey, hydrogen atoms with white colours.

ents in biphenyl would reflect the geometry of the molecule. Low energy conformers of biphenyls **10**, **13** and **14** (both *homochiral* and *meso*) were estimated to adopt a *trans* configuration where the two aliphatic side chains point in opposite directions. In configurationally stable biphenyls **9**, **11** and **12** the two benzene rings lie in perpendicular planes to each other with a dihedral angle of 90° approximately (Table **2**).

The replacement of hydrogen atoms in the aliphatic chain of biphenyl **10** with larger bromine atoms influences the dihedral angle Φ that tunes from 139.0° to 39.8° in biphenyl **14**, the lowest value among those evaluated in the studied biphenyls (Table **2**). In the set of curcumin-biphenyl derivatives, compounds **8**, **17**, **18**, **20**, **21** and **23** adopt a *trans* configuration. Only biphenyl **23** shows a configuration with the

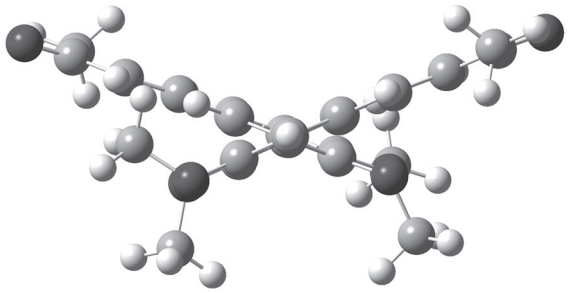


Fig. (4). Molecular structure of low-energy conformer **8**. Oxygen atoms are displayed with dark grey, carbon atoms with grey, hydrogen atoms with white colours.

two benzene rings in orthogonal position and with a dihedral angle Φ approximately 90°.

The lowest-energy conformer of biphenyl **21** adopts a configuration where each benzyl group is nearly parallel to one aromatic ring of the biphenyl scaffold and the two β-diketo enol ester chains are in a linear conformation facing away from the 5,5'-positions (Fig. **3**) and pointing to the same direction. The lowest-energy conformer of biphenyl **8** shows high symmetry with the hydroxylated functionalities pointing at the extremity of volume occupied by the molecule (Fig. **4**). Both compounds **8** and **21** showed conformation with which the molecule would exert more interactions simultaneously with target sites located in different directions compared to those permitted by the other biphenyls (Supplementary Material). Among the studied biphenyls, the highest antiproliferative activity *in vitro* was detected for compounds **8** and **21**.

CONCLUSION

A small collection of stable hydroxylated biphenyls eugenol- and curcumin-analogs has been prepared by straightfoward and sustainable methods. C_2-symmetric hydroxylated biphenyl unit appeared to be a promising scaffold to prepare new antimelanoma agents starting from natural 4-substituted-2-methoxyphenols. Manipulation of the flexible aliphatic side chain at the 5,5'-positions leads to exert small but significant tuning of dihedral angle which bears conformation of the whole molecule and it seems to influence the antitumoral activity. In particular compound **8** and **21** showed a good growth inhibition activity with IC_{50} ranged roughly 1 and 10 μM in the MM cell lines tested, respectively. Preliminary results indicated that unsaturated β-diketo enol ester and α,β-unsaturated ketone chains at the 5,5'-positions of hydroxylated biphenyls appeared to be the key features to prepare new curcumin analogs against melanoma. Further biological assays aimed at evaluating compound **21** and structural derivatives for their *in vivo* antiproliferative activity in nude mice models as well as for their microsomal stability on mouse hepatocyte systems will be the objects of a next article.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

ACKNOWLEDGEMENTS

Authors gratefully thank Sardinia Autonomous Region for providing financial support, L.R. 7 August 2007, n. 7.

SUPPLEMENTARY MATERIAL

Low energy conformers of biphenyls **9-14** and **17**, **18**, **20** and **23**.

Supplementary material is available on the publisher's web site along with the published article.

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Received: July 03, 2014 Revised: September 02, 2014 Accepted: September 15, 2014