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PAPER

Synthesis of new 9-hydroxy-α- and 7-hydroxy-β-pyran naphthoquinones and cytotoxicity against cancer cell lines†

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A synthetic method to obtain α- and β-pyran naphthoquinones **10** and **11** with a hydroxyl substituent on the aromatic ring was developed. Two series of α- and β-pyran naphthoquinones were obtained from the 8-hydroxy-lawsone, and their anticancer properties were evaluated against four tumor cell lines. In general, the new compounds displayed good activity, possibly indicating that these compounds have increased pro-oxidant capacity. The 9-hydroxy-α-lapachone and 7-hydroxy-β-lapachone analogues of the natural products α-lapachone and β-lapachone were successfully produced by this methodology.

Introduction

The International Agency for Research on Cancer (IARC/WHO) predicted that cancer would be the leading cause of death worldwide by the year 2010, and the incidence of cancer is still increasing, particularly in developing countries.[1] Although considerable insight has been gained into the mechanisms by which some chemicals affect cellular growth, and this knowledge has been used for the design of new chemotherapeutic drugs, new and more selective lead compounds are still needed. In this regard, quinones exhibit important cytotoxic activity against cancer cells.[2] Indeed, several quinones, such as doxorubicin, mitomycin, and mitoxantrone, have become medicines that are still used clinically in the therapy of solid cancers.

Lapachol (**1**) is a natural naphthoquinone that occurs in the grain of several wooden trees of the Bignoniaceae family. Lapachol reduces cancer metastasis[3] and acts upon DNA topoisomerases, enzymes that are essential for the integrity of the DNA molecule.[4] Two other important pyranaphthoquinones, the α- (**2**) and β-lapachones (**3**), are isolated as minor components of heartwood from Lapacho trees (Fig. 1). The characteristics of these compounds have been extensively discussed in the literature.[5]

In 1984, Boorstein and Pardee reported that β-lapachone increases the lethality of human fibroblasts.[6] Pursuing this line of research, Pardee and coworkers found that β-lapachone suppressed HIV-1 replication in both acute and chronic infection[7] and also inhibited the activity of topoisomerase by blocking the formation of the topoisomerase I-DNA cleavable complex.[8] Following these seminal works, hundreds of reports on the activity of β-lapachone against various tumor cells were published in just over fifteen years. For example, anticancer activities were reported against human cancer cell lines from leukemia[9] and prostate,[10] malignant glioma,[11] hepatoma,[12] colon,[13] breast,[14] lung[15] and pancreatic cancers.[16] β-Lapachone (**3**) has been reported

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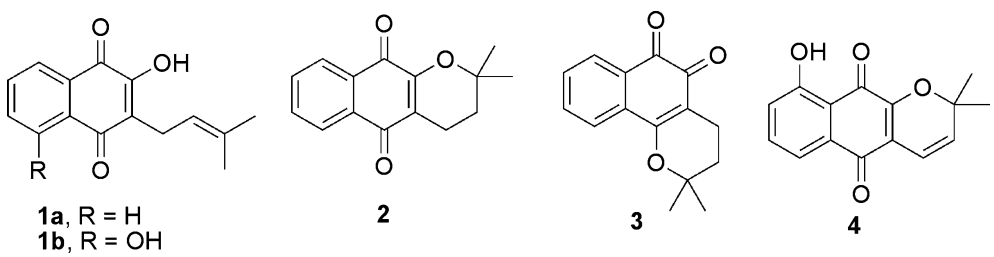

Fig. 1 Important natural naphthoquinones.

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to increase the lethality of human cancer cells in association with other drugs such as taxol,[17] mitomycin C (ovarian)[6] and paclitaxel.[18] Boothman and coworkers discovered another remarkable property of β-lapachone (**3**), an ability to act as a co-adjuvant in killing human cancer cells during radiotherapy treatment. It appears to inhibit sub-lethal radiation damage repair.[19] Since then, this activity has been exploited by several researchers and is currently a medical procedure under patent.[20] A new modality of radiotherapy using gold nanoparticles containing β-lapachone for radiosensitization that improved radiotherapeutic efficacy was reported very recently.[21] A modified cyclodextrin host–guest complex of β-lapachone associated with gemcitabine, named ARQ501, is currently undergoing multiple Phase II clinical trials for use against pancreatic cancer and adenocarcinoma.[22]

Because β-lapachone displays interesting anticancer activities and is readily available from natural sources, it has become a favored structure in medicinal chemistry. Many synthetic routes for obtaining **3** have been developed, and several derivatives have been prepared and biologically evaluated. However, α- and β-lapachones substituted in the aromatic ring have not been well studied because they are difficult to obtain from other natural pyranaphthoquinones by aromatic electrophilic substitution reactions in the aromatic ring. These compounds can be isolated as natural products from several sources. For example, α-caryopterone (**4**), isolated from *Caryopteris clandonensis* (Fig. 1), exhibits molluscicidal and radical scavenging activity.[23] Giles[24] and Oliveira[25] developed syntheses of α-caryopterone (**4**) and, more recently, Padrón[26] reported the synthesis of 5-hydroxy-lapachol (**1b**).

The ability of naphthoquinones to cause damage to DNA and peroxidation of lipids is closely related to the pro-oxidant action of the semiquinone radical. Yoshino and co-workers[27] found that naphthoquinones with a hydroxyl group on the benzene moiety stimulated lipid peroxidation and the formation of adducts with DNA.

In this paper, we report the preparation of α- and β-pyran naphthoquinones **10a–h** and **11a–h**, with a hydroxyl group at position 7 or 9 of the aromatic ring, respectively, *via ortho*-quinone methide intermediates (*o*-QMs) generated *in situ* by Knoevenagel condensation of 2,8-dihydroxy-1,4-naphthoquinone (**9**) with formaldehyde, followed by a hetero Diels–Alder reaction with substituted styrenes in ethanol under microwave irradiation. We also report the cytotoxic activities of these α- and β-lapachone derivatives against four neoplasic cancer cell lines, MDA-MB435 (breast/melanoma), HL-60 (leukemia), HCT-8 (colon) and SF-295 (central nervous system), and toward one normal cell type, murine fibroblast L-929. All cell lines originated from the National Institute of Health, Bethesda, Maryland.

Results and discussion

Chemistry

The first step in the preparation of the desired α- and β-pyran naphthoquinones or α- and β-lapachone derivatives **10a–g** and **11a–g**, was the oxidation of 1,5-naphthalene diol (**5**) with HIO_4 to produce juglone (**6**) in 85% yield. The next step involved a Michael addition of *p*-thiocresol to **6** that produced two isomeric adducts, **7** and **8** (Scheme 1 and Fig. 2). Although a previous report of this reaction[28] only describes the synthesis of product **8**, we obtained a mixture of isomers. Purification of the mixture by column chromatography on silica gel using gradient elution with hexane and toluene furnished adducts **7** and **8** in 40% and 47% yields, respectively.

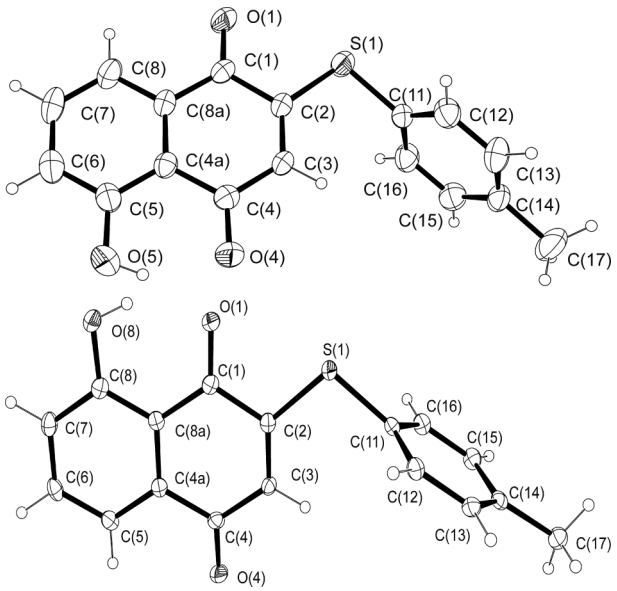
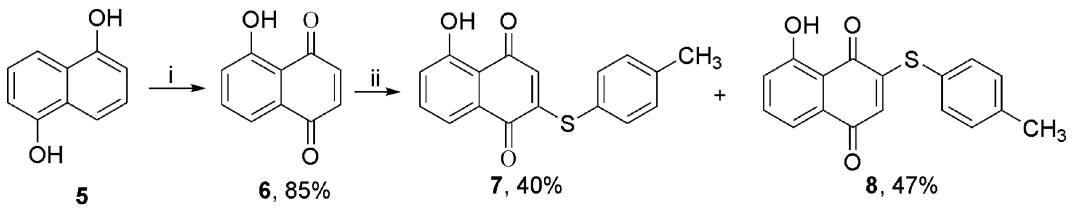


Fig. 2 Ortep drawing of naphthoquinones **7** and **8**, respectively.

It was impossible to differentiate the structures of **7** and **8** on the basis of their ^{1}H and ^{13}C NMR spectra, but we were able to unambiguously determine the structures using X-ray diffraction.

Hydrolysis of **8** under basic conditions produced naphthoquinone **9** in 90% yield. The removal of the *p*-thiocresol group was confirmed by ^{1}H and ^{13}C NMR spectra. The final step in the preparation of the target α-and β-pyran naphthoquinones **10a–h** and **11a–h** was a Knoevenagel reaction followed by hetero Diels–Alder cycloaddition reactions under microwave irradiation



i) HIO_4 2,2 eq; THF/H_2O (1:1)
ii) *p*-thiocresol; ethanol

Scheme 1 Synthesis of 1,4-naphtoquinones **7** and **8**.

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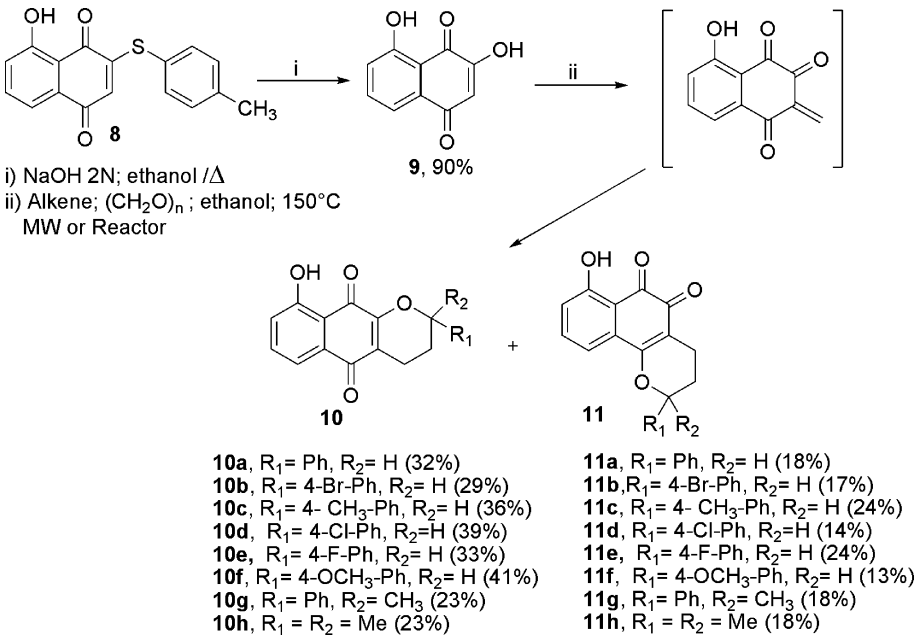
using several styrenes as hetero-dienophiles. This methodology was first reported by Ferreira and Ventura Pinto,[29] in the 1980's, improved by Nair and co-workers,[30] and more recently, expanded by Ferreira and co-workers (Scheme 2).[31] In all reactions, the α-pyran naphthoquinone **10a–h** was the major product in an approximate ratio of 2 : 1. These results are consistent with those described in the literature, which indicate that the α-isomer always forms in greater proportion. These isomers were separated by column chromatography using silica gel and hexane–ethyl acetate eluent mixtures of increasing polarity.

Biological assay

Compounds **10a–h** and **11a–h** were tested *in vitro* against four cancer cell lines and one normal cell line, murine fibroblast L-929, using the MTT assay with doxorubicin as a positive control.[32] Concentrations (in μM) that induced 50% inhibition of cell growth (IC_{50}) are reported in Table 1.

The introduction of the hydroxyl group in the analogues of 9-hydroxy-α-lapachone (**10a,10e**, **10g** and **10h**) increased their activity against all cancer cell lines relative to α-lapachone (**2**),



Scheme 2 Synthesis of pyran naphthoquinones **10a–h** and **11a–h**.

Table 1 Cytotoxic activity expressed as IC_{50} in μM of compounds against cancer cell lines

Compounds		Cancer cell lines[a]				
Entry		MDA-MB435	HL-60	HCT-8	SF295	Erythrocytes (ED_{50} – μg mL^{-1})
1	**Doxo**[b]	0.88 (0.62–1.21)	0.03 (0.02–0.04)	0.06 (0.04–0.08)	0.41 (0.29–0.44)	250
2	**2**	Inactive	Inactive	Inactive	Inactive	250
3	**3**	0.25 (0.16–0,33)	1.65 (1.49–1.78)	0.83 (0.74–0.87)	0.91(0.74–1.11)	250
4	**10a**	6.77 (4.11–9.40)	9.38 (7.08–11.69)	12.44 (9.73–15.14)	Inactive	250
5	**10b**	Inactive	Inactive	Inactive	Inactive	250
6	**10c**	Inactive	Inactive	Inactive	Inactive	250
7	**10d**	Inactive	Inactive	Inactive	Inactive	250
8	**10e**	2.47 (2.07–2.87)	8.73 (5.89–11.56)	10.48 (8.73–12.24)	3.54 (3.02–4.07)	250
9	**10g**	6.80 (4.40–9.21)	12.05 (6.27–17.82)	13.84 (11.51–16.17)	ND	250
10	**10h**	2.90 (2.24–3.76)	6.03 (5.34–6.73)	6.72 (5.57–7.86)	5.63 (4.49–6.78)	250
11	**11a**	0.11 (0.11–0.12)	1.30 (1.11–1.50)	1.40 (1.14–1.73)	2.15 (1.86–2.51)	250
12	**11b**	0.15 (0.10–0.20)	3.84 (2.28–6.52)	3.30 (1.63–6.67)	0.99 (0.78–1.22)	250
13	**11c**	0.15 (0.09–0.22)	3.28 (1.56–6.84)	3.28 (2.25– 4.71)	2.34 (1.78–3.06)	250
14	**11d**	0.17 (0.15–0.19)	3.40 (2.52–4.61)	2.49 (2.11–2.90)	2.84 (2.26–3.55)	250
15	**11e**	0.21 (0.20–0.27)	3.21 (2.65–3.85)	2.81 (2.31–3.39)	3.21 (2.25–4.56)	250
16	**11f**	0.53 (0.35–0.80)	2.85 (1.28–6.27)	5.88 (2.59–13.29)	6.69 (3.75–11.98)	250
17	**11g**	0.47 (0.46–0.53)	2.56 (2.15–3.03)	3.06 (2.72–3.40)	4.71 (3.78–5.93)	250
18	**11h**	0.31 (0.27–0.36)	1.94 (1.63–2.28)	2.17 (2.01–2.32)	2.52 (2.13–3.02)	250

[a] Data are presented as IC_{50} values and 95% confidence intervals obtained by nonlinear regression for all cell lines from three independent experiments. [b] Doxorubicin (Doxo) was used as positive control. Only compounds with an IC_{50} value lower than 5 μg mL^{-1} for at least one cell line were considered active.

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which is inactive, but they were less active than the 7-hydroxy-β-lapachones (**11a–h**). Nevertheless, it seems that these compounds were more pro-oxidant than the parent structure.

The introduction of the hydroxyl group at C-7 of the pyran naphthoquinones promoted changes in the pattern of activity of these substances compared with β-lapachone (**3**) and doxorubicin. In general, the β-pyran naphthoquinones **11a–h** were more active than doxorubicin on melanoma cells (**MDA-MB435**). The presence of halogens on **11b**, **11d** and **11e** increased the selectivity for **MDA-MB435** without any significant difference between the three compounds. With the exception of 7-hydroxy-β-lapachone (**11h**, entry 18), which was less active than β-lapachone (**3**, entry 3) against all cell lines, the β-pyran naphthoquinones **11a–h** (entries 11–18) were more active and selective against melanoma cancer cells (**MDA-MB435**) than β-lapachone (**3**) and doxorubicin. Compound **11a** was particularly effective, displaying 8-fold greater activity than doxorubicin. None of the compounds exhibited lytic effects against mouse erythrocytes.

Conclusion

A synthetic method to obtain α- and β-pyran naphthoquinones **10a–h** and **11a–h** with a hydroxyl substituent on the aromatic ring was developed. Two series of α- and β-pyran naphthoquinones were obtained from the 8-hydroxy-lawsone, and their anticancer properties were evaluated against four tumor cell lines. In general, the series of 9-hydroxy-α-lapachones displayed greater activity than α-lapachone (**2**), possibly indicating that these compounds have increased pro-oxidant capacity. The derivatives of 7-hydroxy-β-lapachone (**11a–h**) showed excellent results against melanoma cancer cells (**MDA-MB435**). Compounds **11a** (IC_{50} 0.11 μM), **11b** (IC_{50} 0.15 μM), **11c** (IC_{50} 0.15 μM), **11d** (IC_{50} 0.17 μM) and **11f** (IC_{50} 0.23 μM) were particularly effective. These results demonstrate that the introduction of a hydroxyl group on the aromatic ring increased the selectivity and activity of these compounds for **MDA-MB435** cells and suggest that other substituents may also increase these activities if located on other positions of the aromatic ring. Finally, the 9-hydroxy-α-lapachone (**10h**) and 7-hydroxy-β-lapachone (**11h**) analogues of the natural products α-lapachone (**2**) and β-lapachone (**3**) were successfully produced by this methodology.

Experimental

Chemical synthesis

General remarks. Melting points were obtained on a Fisher–Johns melting point apparatus and are uncorrected. Analytical grade solvents were used, and the solvents were previously purified as described in the literature.[33] Column chromatography was performed on silica-gel (Acros Organics 0.035–0.070 mm, pore diameter *ca.* 6 nm). Infrared spectra were recorded on an ABB FTLA2000-100 spectrometer. ^{1}H NMR spectra were collected in $CDCl_3$ on a Varian Unity Plus 300 instrument. Elemental analysis was performed with a Perkin-Elmer CHN 2400. X-ray diffraction of compound 7 was performed on a Bruker-Kappa-CCD diffractometer using Mo-Kα radiation (λ = 0.71069 Å) at room temperature, and compound 8 was analyzed on a Oxford Diffraction Gemini A Ultra using Cu-Kα radiation (λ = 1.5418 Å) at 130(2)K. Compounds 10a–g and 11a–g were prepared using a CEM microwave irradiation Discover 1. Compounds 10 h and 11 h were prepared in a Berghof High-Pressure Reactor BR-300. Compound **6** was prepared according to literature data.[34]

8-hydroxy-2-(p-tolylthio)naphthalene-1,4-dione (8) and 5-hydroxy-2-(p-tolylthio)naphthalene-1,4-dione (7)

A round-bottom flask equipped with a magnetic stirring bar was loaded with **6** (5.4 mmol) dissolved in ethyl alcohol (40 mL). Next, 4-methylbenzenethiol (5.4 mmols) was dissolved in ethyl alcohol (10 mL) and added to the reaction mixture. The reaction proceeded for 4 h, after which the ethanol was removed under reduced pressure. The residual crude product was purified *via* silica-gel chromatography, using a gradient mixture of hexane and toluene to yield **7** (40%) and **8** (47%). The physical characteristics of these compounds were identical to previous reports.[28]

Crystal structure determinations

Crystal data **7**: $C_{17}H_{12}O_3S$, Mr = 296.34, monoclinic, $P2_1/n$, a = 9.713 (2)Å, b = 11.179 (2)Å, c = 13.663 (3)Å, β = 109.59(3)°, V = 1397.7(6)Å^3, Z = 4, T = 293(2) K, μ = 0.24 mm^{-1}, 8729 reflections measured, 2473 unique (R_{int} = 0.0920). The final $R_1(F^2)$ was 0.0509 for all data. CCDC reference number 811450.

Crystal data **8**: $C_{17}H_{12}O_3S$, Mr = 296.34, monoclinic, $P2_1/c$, a = 8.9234(7)Å, b = 8.6499(7)Å, c = 17.8425(8)Å, β = 103.82(1)°, V = 1337.3(2)Å^3, Z = 4, T = 130(2) K, μ = 2.21 mm^{-1}, 3868 reflections measured, 1872 unique (R_{int} = 0.020). The final $R_1(F^2)$ was 0.0345 for all data. CCDC reference number 811449.

2,8-dihydroxy-1,4-naphthoquinone (9)

A round-bottom flask equipped with a magnetic stirring bar was loaded with **8** (1.5 mmol) dissolved in ethyl alcohol (24 mL) and an aqueous solution of NaOH (2 N, 12 mL). The mixture was heated under reflux for 3 h. Water (30 mL) was added, and the mixture was cooled in ice bath and neutralized with 4 N H_2SO_4. The ethanol was removed under reduced pressure, and the mixture was extracted with ethyl acetate. The organic layer was washed with water, dried over anhydrous sodium sulfate, filtered and concentrated under reduced pressure. The residual crude product was purified *via* silica-gel chromatography, using a gradient mixture of hexane and ethyl acetate to yield **9** in 90%. The physical characteristics of **9** were identical to previous reports.[35]

General procedures for preparing 10a–g and 11a–g

A 10 mL microwave tube was loaded with **9** (2.6 mmol), paraformaldehyde (8 mmols), the appropriate styrene (3 mmols) and anhydrous ethanol (5 mL) and irradiated for 15 min. The internal temperature reached 150 °C. The solvent was evaporated under reduced pressure, and the crude mixture was extracted with ethyl acetate (30 mL). The organic layer was washed with water (3 × 20 mL), dried over anhydrous sodium sulfate, filtered and then concentrated under reduced pressure. The residual solid product was purified by column chromatography on silica-gel and eluted with an increasing polarity gradient mixture of hexane and ethyl acetate (9/1 to 7/3).

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7-Hydroxy-2-phenyl-3,4-dihydro-2*H*-benzo[h]chromene-5,6-dione (11a)

The reaction produced **11a** in 18% as a red solid. m.p.: 153–155 °C. IR (KBr) ν_{max}/cm^{-1} 1583 and 1638 (C═O); 3399 (OH);^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.99–2.13 (1H, m, H-3a); 2.28–2.37 (1H, m, H-3b); 2.53–2.64 (1H, m, H-4a); 2.70–2.79 (1H, m, H-4b); 5.25 (1H, dd, J = 10.3, 2.8, H-2); 7.07 (1H, dd, J = 8.4, 0.9, H-8); 7.37 (1H, dd, J = 7.4, 0.9, H-10); 7.39–7.48 (5H, m, 2-phenyl); 7.53 (1H, dd, J = 8.7, 7.4, H-9); 11.99 (1H, s, OH). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 18.4 (C-4); 28.3 (C-3); 80.1 (C-2); 113.4 (C-4a); 114.0 (C-6a); 116.9 (C-10); 121.6 (C-8); 125.8 (C-2′ and C-3′-phenyl); 128.5 (C-6′-phenyl); 128.7 (C-4′ and C-5′-phenyl); 131.8 (C-10a); 138.0 (C-9); 139.3 (C-1′-phenyl); 162.4 (C-7); 164.4 (C-10b); 178.1 (C-5); 182.8 (C-6). Anal. Calcd for $C_{19}H_{14}O_4$: C, 74.50; H, 4.61. Found: C, 74.84; H, 4.75.

9-Hydroxy-2-phenyl-3,4-dihydro-2*H*-benzo[g]chromene-5,10-dione (10a)

The reaction produced **10a** in 32% as an orange solid. m.p.: 122–124 °C. IR (KBr) ν_{max}/cm^{-1} 1603 and 1636 (C═O); 3416 (OH).^{1}H NMR ($CDCl_3$, 300 MHz): δ 2.04–2.12 (1H, m, H-3a); 2.30–2.35 (1H, m, H-3b); 2.62–2.69 (1H, m, H-4a); 2.72–2.78 (1H, m, H-4b); 5.20 (1H, dd, J = 9.6, 2.6, H-2); 7.21 (1H, dd, J = 8.1, 1.4, H-6); 7.34–7.43 (5H, m, 2-phenyl); 7.59 (1H, dd, J = 8.1, 7.6, H-7); 7.63 (1H, dd, 7.6, 1.4, H-8); 11.84 (1H, s, OH). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 12.7 (C-3); 27.8 (C-4); 79.2 (C-2); 114.1 (C-4a); 118.8 (C-6); 122.5 (C-9a); 123.7 (C-8); 125.8 (C-2′ and C-3′-phenyl); 128.4 (C-6′-phenyl); 128.7 (C-1′-phenyl); 128.7 (C-4′ and C-5′-phenyl); 131.9 (C-5a); 136.6 (C-7); 155.0 (C-9); 161.6 (C-10a); 183.3 (C-5); 184.3 (C- 10). Anal. Calcd for $C_{19}H_{14}O_4$: C, 74.50; H, 4.61. Found: C, 74.51; H, 4.47.

2-(4-bromophenyl)-7-hydroxy-3,4-dihydro-2*H*-benzo[h]chromene-5,6-dione (11b)

The reaction produced **11b** in 17% as a red solid. m.p.: decompose over 160 °C. IR (KBr) ν_{max}/cm^{-1} 1589 e 1642 (C═O).^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.95–2.10 (1H, m, H-3a); 2.26–2.35 (1H, m, H-3b); 2.52–2.63 (1H, m, H-4a); 2.70–2.79 (1H, m, H-4b); 5.20 (1H, dd, J = 10.3; 2.6, H-2); 7.08 (1H, dd, J = 8.6, 1.0, H-8); 7.27–7.31 (2H, m, H-*meta*-phenyl); 7.32 (1H, dd, J = 7.6, 1.0, H-10); 7.52 (1H, dd, J = 8.3, 7.3, H-9); 7.55–7.60 (2H, m, H-*ortho*-phenyl). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 18.3 (C-3); 28.2 (C-4); 79.4 (C-2); 114.0 (C-4a); 116.8 (C-10); 121.8 (C-8; C-2′ and C-3′-phenyl); 122.5 (C-6a and C-6′-phenyl); 132.0 (C-4′ and C-5′-phenyl); 138.0 (C-9); 138.3 (C-10a); 139.0 (C-1′-phenyl); 162.1 (C-7); 164.5 (C-10b); 178.1 (C-5); 182,6 (C-6). Anal. Calcd for $C_{19}H_{13}BrO_4$: C, 59.24; H, 3.40. Found: C, 60.38; H, 3.47.

2-(4-bromophenyl)-9-hydroxy-3,4-dihydro-2*H*-benzo[g] chromene-5,10-dione (10b)

The reaction produced **10b** in 29% as an orange solid. m.p.: 186–189 °C. IR (KBr) ν_{max}/cm^{-1} 1615 and 1643 (C═O); 3192 (OH). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.96–2.09 (1H, m, H-3a); 2.26–2.35 (1H, m, H-3b); 2.58–2.68 (1H, m, H-4a); 2.70–2.81 (1, m, H-4b); 5.14 (1H, dd, J = 9.6, 2.6, H-2); 7.21 (1H, dd, J = 7.6, 2.0, H-6); 7.27–7.31 (2H, m, H-*meta*-phenyl); 7.52–7.56 (2H, m, H-*ortho*-phenyl); 7.60 (1H, dd, J = 7.6, 7.6, H-7); 7.64 (1H, dd, J = 7.6, 2.0, H-8); 11.81 (1H, s, OH). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 18.7 (C-3); 27.8 (C-4); 78.5 (C-2); 113.9 (C-4a); 118.8 (C-6); 122.4 (C-9a); 122.5 (C-6′-phenyl); 123.7 (C-8); 127.5 (C-2′ and C-3′-phenyl); 131.9 (C-5a); 131.9 (C-4′and C-5′-phenyl); 136.7 (C-7); 143.4 (C-1′-phenyl); 154.7 (C-9); 161.6 (C-10a); 183.1 (C-5); 184.1 (C-10). Anal. Calcd for $C_{19}H_{13}BrO_4$: C, 59.24; H, 3.40. Found: C, 60.47; H, 3.58.

7-Hydroxy-2-*p*-tolyl-3,4-dihydro-2*H*-benzo[h]chromene-5,6-dione (11c)

The reaction produced **11c** in 24% as a red solid. m.p.: 165–167 °C. IR (KBr) ν_{max}/cm^{-1} 1585 and 1639 (C═O); 3405 (OH).^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.99–2.12 (1H, m, H-3a); 2.25–2.34 (1H, m, H-3b); 2.40 (3H, s, CH_3); 2.52–2.63 (1H, m, H-4b); 2.70–2.79 (1H, m, H-4a); 5.21 (1H, dd, J = 10.3, 2.6, H-2); 7.06 (1H, dd, J = 8.8, 1.2, H-8); 7.23–7.32 (4H, m, 2-*p*-tolyl); 7.35 (1H, dd, J = 7.6, 1.2, H-5); 7.51 (1H, dd, J = 8.8, 7.6, H-9); 11.99 (1H, s, OH). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 18.4 (C-3); 28.2 (C-4); 21.2 (CH_3); 80.1 (C-2); 113.4 (C-4a); 114.0 (C-6a); 116.9 (C-10); 121.6 (C-8); 125.8 (C-2′ and C-3′-phenyl); 129.4 (C-4′ and C-5′-phenyl); 131.8 (C-10a); 136.3 (C-1′-phenyl); 138.0 (C-9); 138.5 (C-6′-phenyl); 162.5 (C-7); 164.4 (C-10b); 178.1 (C-5); 182.8 (C-6). Anal. Calcd for $C_{20}H_{16}O_4$: C, 74.99; H, 5.03. Found: C, 74.99; H, 5.32.

9-Hydroxy-2-*p*-tolyl-3,4-dihydro-2*H*-benzo[g]chromene-5,10-dione (10c)

The reaction produced **10c** in 36% as an orange solid. m.p.: 177–179 °C. IR (KBr) ν_{max}/cm^{-1} 1619 and 1644 (C═O); 3433 (OH). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 2.01–2.14 (1H, m, H-3a); 2.25–2.34 (1H, m, H-3b); 2.37 (3H, s, CH_3); 2.57–2.67 (1H, m, H-4a); 2.69–2.79 (1H, m, H-4b); 5.17 (1H, dd, 9.6, 2.4, H-2); 7.19–7.22 (2H, m, H-*ortho*-phenyl); 7.19–7.22 (1H, m, H-6); 7.27–7.30 (2H, m, H-*meta*-phenyl); 7.56–7.58 (1H, m, H-7); 7.61–7.64 (1H, m, H-8); 11.84 (1H, s, OH). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 18.7 (C-3); 21.1 (CH_3); 27.6 (C-4); 79.2 (C-2); 114.0 (C-4a); 118.7 (C-6); 122.4 (C-9a); 123.6 (C-8); 125.8 (C-2′ and C-3′-phenyl); 128.8 (C-4′ and C-5′-phenyl); 129.3 (C-6′-phenyl); 131.9 (C-5a); 136.1 (C-7); 138.2 (C-1′-phenyl); 155.0 (C-9); 161.6 (C-10a); 183.3 (C-5); 184.3 (C-10). Anal. Calcd for $C_{20}H_{16}O_4$: C, 74.99; H, 5.03. Found: C, 74.33; H, 4.99.

2-(4-chlorophenyl)-7-hydroxy-3,4-dihydro-2*H*-benzo[h] chromene-5,6-dione (11d)

The reaction produced **11d** in 14% as a red solid. m.p.: 140–142 °C. IR (KBr) ν_{max}/cm^{-1} 1584 and 1638 (C═O); 3412 (OH). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.96–2.09 (1H, m, H-3a); 2.26–2.35 (1H, m, H-3b); 2.53–2.64 (1H, m, H-4a); 2.70–2.79 (1H, m, H-4b); 5.22 (1H, dd, J = 10.2, 2.6, H-2); 7.08 (1H, dd, J = 8.6, 1.0, H-8); 7.34 (1H, dd, J = 7.6, 1.0, H-10); 7.34–7.38 (2H, m, H-*meta*-phenyl); 7.40–7.45(2H, m, H-*ortho*-phenyl); 7.53 (1H, dd, J = 8.6, 7.6, H-9); 11.98 (1H, s, OH). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 18.3 (C-4); 28.2 (C-3); 79.4 (C-2); 113.3 (C-4a); 114.0 (C-6a); 116.8 (C-10); 121.7 (C-8); 127.2 (C-4′ and C-5′-phenyl); 129.0 (C-2′ and C-3′-phenyl); 131.6 (C-6′-phenyl); 134.4 (C-10a); 137.8 (C-1′-phenyl); 138.0 (C-9); 162.1 (C-7); 164.5 (C-10b); 178.1 (C-5); 182.6

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(C-6). Anal. Calcd for $C_{19}H_{13}ClO_4$: C, 66.97; H, 3.85. Found: C, 66.62; H, 3.98.

2-(4-Chlorophenyl)-9-hydroxy-3,4-dihydro-2*H*-benzo[g]chromene-5,10-dione (10d)

The reaction produced **10d** in 39% as an orange solid. m.p.: 167–169 °C. IR (KBr) ν_{max}/cm^{-1} 1610 and 1641 (C═O); 3437 (OH). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.96–2.10 (1H, m, H-3a); 2.27–2.36 (1H, m, H-3b); 2.58–2.70 (1H, m, H-4a); 2.72–2.81 (1H, m, H-4b); 5.16 (1H, dd, J = 9.6, 2.6, H-2); 7.22 (1H, dd, J = 7.6, 2.0, H-6); 7.33–7.41 (4H, m, H-Ar); 7.60 (1H, dd, J = 7.9, 7.3, H-7); 7.64 (1H, dd, J = 7.3, 2.0, H-8); 11.81 (1H, s, OH). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 18.7 (C-3); 27.8 (C-4); 78.5 (C-2); 113.9 (C-4a); 118.8 (C-6); 122.5 (C-9a); 123.7 (C-8); 127.2 (C-2′ and C-3′-phenyl); 128.9 (C-4′ and C-5′-phenyl); 131.8 (C-6′-phenyl); 134.3 (C-5a); 136.7 (C-7); 137.6 (C-1′-phenyl); 154.7 (C-9); 161.6 (C-10a); 183.2 (C-5); 184.3 (C-10). Anal. Calcd for $C_{19}H_{13}ClO_4$: C, 66.97; H, 3.85. Found: C, 66.94; H, 4.12.

2-(4-Fluorophenyl)-7-hydroxy-3,4-dihydro-2*H*-benzo[h]chromene-5,6-dione (11e)

The reaction produced **11e** in 24% as a red solid. m.p.: 181–183 °C. IR (KBr) ν_{max}/cm^{-1} 1584 and 1640 (C═O); 3067 (OH). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.97–2.11 (1H, m, H-3a); 2.35–2.27(1H, m, H-3b); 2.52–2.64- (1H, m, H-4a); 2.72–2.80 (1H, m, H-4b); 5.22 (1H, dd, J = 10.2, 2.3, H-2); 7.08 (1H, dd, J = 8.6, 1.0, H-8); 7.10–7.18 (2H, m, H-*meta*-phenyl); 7.34 (1H, dd, J = 7.6, 1.0, H-10); 7.37–7.43 (2H, m, H-*ortho*-phenyl); 7.53 (1H, dd, J = 8.6, 7.6, H-9); 11.98 (1H, s, OH). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 18.4 (C-3); 28.3 (C-4); 79.5 (C-2); 113.3 (C-4a); 114.0 (C-6a); 115.7 (J = 21.0, C-4′ and C-5′-phenyl); 116.8 (C-10); 121.7 (C-8); 127.7 (J = 7.7, C-2′ and C-3′-phenyl); 135.1 (C-10a); 135.1 (C-1′-phenyl); 138.0 (C-9); 161.0 (C-7); 162.7 (J = 247.7, C-6′-phenyl); 164.5 (C-10b); 178.1 (C-5); 182.7 (C-6). Anal. Calcd for $C_{19}H_{13}FO_4$: C, 70.37; H, 4.04. Found: C, 70.18; H, 4.39.

2-(4-Fluorophenyl)-9-hydroxy-3,4-dihydro-2*H*-benzo[g]chromene-5,10-dione (10e)

The reaction produced **10e** in 33% as an orange solid. m.p.: 138–140 °C. IR (KBr) ν_{max}/cm^{-1} 1617 and 1640 (C═O); 3420 (OH). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.98–2.11 (1H, m, H-3a); 2.27–2.35 (1H, m, H-3b); 2.58–2.70 (1H, m, H-4a); 2.74–2.83 (1H, m, H-4b); 5.15 (1H, dd, J = 9.9, 2.6, H-2); 7.06–7.14 (2H, m, H-*meta*-phenyl); 7.21 (1H, J = dd, 7.6, 2.0, H-6); 7.35–7.42 (2H, m, H-*ortho*-phenyl); 7.59 (1H, dd, J = 7.6, 7.6, H-7); 7.64 (1H, dd, J = 7.6, 2.0, H-8); 11.81 (1H, s, OH). ^{13}C NMR ($CDCl_3$, 75 MHz): δ18.8 (C-3); 27.9 (C-4); 78.6 (C-2); 113.9 (C-4a); 115.7 (J = 21.0; C-4′ and C-5′-phenyl); 118.8 (C-6); 122.4 (C-9a); 123.7 (C-8); 127.7 (J = 7.7, C-2′ and C-3′-phenyl); 131.8 (C-1′-phenyl); 134.9 (C-5a); 136.6 (C-7); 154.7 (C-9); 161.6 (C-10a); 162.6 (J = 246.6, C-6′-phenyl); 183.2 (C-5); 184.3 (C-10). Anal. Calcd for $C_{19}H_{13}FO_4$: C, 70.37; H, 4.04. Found: C, 70.23; H, 4.32.

7-Hydroxy-2-(4-methoxyphenyl)-3,4-dihydro-2*H*-benzo[h]chromene-5,6-dione (11f)

The reaction produced **11f** in 13% as a red solid. m.p.: 128–130 °C. IR (KBr) ν_{max}/cm^{-1} 1584 and 1638 (C═O); 3270 (OH). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 2.01–2.09 (1H, m, H-3a); 2.26–2.29 (1H, m, H-3b); 2.52–2.59 (1H, m, H-4a); 2.72–2.76 (1H, m, H-4b); 3.84 (3H, s, CH_3); 5.18 (1H, dd, J = 10.3, 2.3, H-2); 6.95–6.97 (2H, m, H-*meta*-phenyl); 7.04 (1H, dd, J = 8.8, 1.2, H-8); 7.31–7.35 (2H, m, H-*ortho*-phenyl); 7.33 (1H, dd, J = 7.3, 1.2, H-10); 7.49 (1H, dd, J = 8.5, 7.6, H-9); 11.96 (1H, s, OH). ^{13}C NMR ($CDCl_3$, 75 MHz): δ18.6 (C-4); 28.1 (C-3); 53.3 (CH_3); 80.0 (C-2); 113.3 (C-4a); 114.0 (C-6a); 114.1 (C-4′and C-5′-phenyl); 116.9 (C-10); 121.6 (C-8); 127.4 (C-2′ and C-3′-phenyl); 131.3 (C-1′-phenyl); 131.8 (C-10a); 138.0 (C-9); 159.8 (C-6′-phenyl); 162.6 (C-7); 164.4 (C-10b); 178.1 (C-5); 182.8 (C-6). Anal. Calcd for $C_{20}H_{16}O_5$: C, 71.42; H, 4.79. Found: C, 71.21; H, 4.95.

9-Hydroxy-2-(4-methoxyphenyl)-3,4-dihydro-2*H*-benzo[g]chromene-5,10-dione (10f)

The reaction produced **10f** in 41% as an orange solid. m.p.: 143–145 °C. IR (KBr) ν_{max}/cm^{-1} 1618 and 1643 (C═O). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 2.01–2.14 (1H, m, H-3a); 2.24–2.33 (1H, m, H-3b); 2.57–2.69 (1H, m, H-4a); 2.73–2.82 (1H, m, H-4b); 3.82 (3H, s, CH_3); 5.15 (1H, dd, J = 9.9, 2.6,H-2); 6.90–6.95 (2H, m, H-*meta*-phenyl); 7.21 (1H, dd, J = 7.8, 1.9, H-6); 7.31–7.35 (2H, m, H-*ortho*-phenyl); 7.59 (1H, dd, J = 7.8, 7.5, H-7); 7.63 (1H, dd, J = 7.5, 1.9, H-8); 11.83 (1H, s, OH). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 18.9 (C-3); 27.7 (C-4); 53.3 (CH_3); 79.2 (C-2); 114.1 (C-4a); 114.2 (C-4′ and C-5′-phenyl); 118.8 (C-6); 122.4 (C-10a); 123.6 (C-8); 127.4 (C-2′ and C-3′-phenyl); 131.2 (C-1′-phenyl); 132.1 (C-6a); 136.6 (C-7); 155.2 (C-9); 159.8 (C-6′-phenyl); 161.7 (C-10a); 183.4 (C-5); 184.4 (C-10). Anal. Calcd for $C_{20}H_{16}O_5$: C, 71.42; H, 4.79. Found: C, 71.56; H, 5.08.

7-Hydroxy-2-methyl-2-phenyl-3,4-dihydro-2*H*-benzo[h]chromene-5,6-dione (11g)

The reaction produced **11g** in 18% as a red solid. m.p.: 161–162 °C. IR (KBr) ν_{max}/cm^{-1} 1593 and 1640 (C═O); 3415 (OH). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.76 (3H, s, CH_3); 2.04–2.13 (1H, m, H-3a); 2.16–2.20 (1H, m, H-3b); 2.45–2.53 (1H, m, H-4a); 2.62–2.71 (1H, m, H-4b); 7.27–7.35 (5H, m, 2-phenyl); 7.18–7.23 (1H, m, H-9); 7.56–7.58 (2H, m, H-8 and H-10); 11.90 (1H, s, OH). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 17.0 (C-3); 29.7 (CH_3); 31.1 (C-4); 81.7 (C-2); 114.1 (C-4a); 118.7 (C-10); 122.6 (C-6a); 123.6 (C-8); 124.1 (C-6′-phenyl); 127.5 (C-2′ and C-3′-phenyl); 128.8 (C-4′ and C-5′-phenyl); 131.9 (C-10a); 136.5 (C-9); 143.4 (C-1′-phenyl); 154.0 (C-8); 161.5 (C-10b); 183.4 (C-5); 184.5 (C-6). Anal. Calcd for $C_{20}H_{16}O_4$: C, 74.99; H, 5.03. Found: C, 74.54; H, 5.36.

9-Hydroxy-2-methyl-2-phenyl-3,4-dihydro-2*H*-benzo[g]chromene-5,10-dione (10g)

The reaction produced **10g** in 23% as an orange solid. m.p.: 126–127 °C. IR (KBr) ν_{max}/cm^{-1} 1611 and 1641 (C═O); 3428 (OH). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.75 (3H, s, CH_3); 2.01–2.10 (1H, m, H-3a); 2.15–2.26 (1H, m, H-3b); 2.39–2.46 (1H, m, H-4a); 2.56–2.64 (1H, m, H-4b); 7.31–7.35 (5H, m, 2-phenyl); 7.11 (1H, dd, J = 8.3, 1.3, H-8); 7.49 (1H, dd, J = 8.5, 7.6, H-7); 7.62 (1H, dd, J = 7.9, 7.6, H-6); 11.99 (1H, s, OH). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 16.3 (C-3); 29.3 (C-CH_3); 31.8 (C-4); 82.7 (C-2); 113.5 (C-4a); 114.0 (C-9a); 116.6 (C-6); 121.7 (C-8); 124.0 (C-6′-phenyl); 127.6 (C-2′ and C-3′-phenyl); 128.8 (C-4′ and C-5′-phenyl); 132.0 (C-5a);

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138.1 (C-7); 143.5 (C-1′-phenyl); 161.3 (C-9); 164.5 (C-10a); 178.0 (C-5); 182.9 (C-10). Anal. Calcd for $C_{20}H_{16}O_4$: C, 74.99; H, 5.03. Found: C, 74.47; H, 5.36.

General procedures for preparing 10h and 11h

A 600 mL reactor was loaded with **9** (2.6 mmol), paraformaldehyde (8 mmol), isobutylene and ethyl alcohol (150 mL). The reaction was heated at 150 °C for 3 h, after which the ethyl alcohol was evaporated under reduced pressure. The crude mixture was extracted with ethyl acetate, and the organic layer was washed with water, dried over anhydrous sodium sulfate, filtered and concentrated under reduced pressure. The residual crude product was purified *via* silica-gel chromatography, using a gradient mixture of hexane and ethyl acetate.

7-hydroxy-2,2-dimethyl-3,4-dihydro-2*H*-benzo[h]chromene-5,6-dione (11h)

The reaction produced **11h** in 23% as a red solid. m.p.: 150–152 °C. IR (KBr) ν_{max}/cm^{-1} 1579 and 1639 (C═O); 3424 (OH). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.45 (6H, s, CH_3); 1.84 (1H, dd, 6.6, 6.6, H-3); 2.45 (1H, dd, J = 6.6, 6.6, H-4); 7.05 (1H, dd, J = 8.6, 1.0, H-8); 7.34 (1H, dd, J = 7.6, 1.0, H-10); 7.53 (1H, dd, J = 8.6, 7.6, H-9); 11.98 (1H, s, OH). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 16.1 (C-3); 26.7 (CH_3); 31.5 (C-4); 79.2 (C-2); 112.7 (C-4a); 116.7 (C-10); 113.5 (C-6a); 121.4 (C-8); 132.3 (C-10a); 137.8 (C-9); 161.5 (C-7); 164.3 (C-10b); 178.1 (C-5); 183.1 (C-6). Anal. Calcd for $C_{15}H_{14}O_4$: C, 69.76; H, 5.46. Found: C, 69.34; H, 6.05.

9-hydroxy-2,2-dimethyl-3,4-dihydro-2*H*-benzo[g] chromene-5,10-dione (10h)

The reaction produced **10h** in 40% as an orange solid. m.p.: 178–180 °C. IR (KBr) ν_{max}/cm^{-1} 1608 and 1640 (C═O); 3411 (OH). ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.44 (6H, s, CH_3); 1.82 (1H, dd, 6.6, 6.6, H-3); 2.61(1H, dd, J = 6.6, 6.6, H-4); 7.19 (1H, dd, J = 7.8, 1.9, H-7); 7.56 (1H, dd, J = 7.8, 0.3, H-8); 7.60 (1H, dd, J = 1.9, 0.3 H-6); 11.84 (1H, s, OH). ^{13}C NMR ($CDCl_3$, 75 MHz): δ 16.8 (C-3); 26.5 (CH_3); 31.4 (C-4); 78.3 (C-2); 114.2 (C-4a); 118.6 (C-6); 122.4 (C-9a); 123.4 (C-8); 132.1 (C-5a); 136.4 (C-7); 154.1 (C-9); 161.6 (C-10a); 183.3 (C-5); 184.8 (C-10). Anal. Calcd for $C_{15}H_{14}O_4$: C, 69.76; H, 5.46. Found: C, 69.38; H, 5.73.

Biology

Cytotoxicity against cancer cell lines. Compounds (0.009–5 μg mL^{-1}) were tested for cytotoxic activity against four cancer cell lines: SF-295 (glioblastoma), HCT-8 (colon), MDA-MB435 (melanoma), and HL60 (leukemia) (National Cancer Institute, Bethesda, MD). All cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, and 100 g mL^{-1} streptomycin at 37 °C in a 5% CO_2 atmosphere. Each compound was dissolved with DMSO to a concentration of 1 mg mL^{-1}. The final concentration of DMSO in the culture medium was kept constant, below 0.1% (v/v). Compounds were incubated with the cells for 72 h. The negative control was incubated with an equal amount of DMSO alone (0.001% in the highest concentration). Doxorubicin (0.18–1.06 M) was used as a positive control. The cell viability was determined by reduction of the yellow dye 3-(4,5-dimethyl-2-thiazol)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) to a blue formazan product, as described by Mosmann.[32]

Cell Membrane Disruption. This test was performed in 96-well plates using a 2% mouse erythrocyte suspension in 0.85% NaCl containing 10 mM $CaCl_2$, following the method previously described.[36] The compounds were tested at concentrations ranging from 1.95 to 250 μg mL^{-1}. After incubation at room temperature for 30 min and centrifugation, the supernatant was removed, and the liberated hemoglobin was measured spectrophotometrically at 540 nm. DMSO was used as a negative control, and Triton X-100 (1%) was used as a positive control. After incubation at room temperature for 60 min and centrifugation, the supernatant was removed, and the liberated hemoglobin was measured spectrophotometrically at 540 nm. The EC_{50} is the calculated effective concentration that induced 50% of the lysis induced by Triton X-100.

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