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A novel, broad-spectrum antitumor compound containing the 1-hydroxycyclohexa-2,5-dien-4-one group: The disclosure of a new antitumor pharmacophore in protoapigenone 1

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ABSTRACT

The synthesis of a new compound **9** containing the 4-hydroxy-2,5-cyclohexadien-1-one system, a key elements toward elucidation of the protoapigenone **1** antitumor pharmacophore, was described. The compound showed potent in vitro antitumor potency with low micromolar IC_{50}'s against breast, ovarian, prostate, liver, pancreas, and blood cancer cell lines tested and could inhibit tumor growth in vivo but no significant impairment of hematopoiesis or immune function was observed. The minimum structural pharmacophore of **1** has now been refined.

There has been considerable interest in both the chemistry and biological activity of the ferns over the past decades. Many types of flavonoids have been discovered from different fern species and some of these have shown great promise in cancer therapy.[1–4] Protoapigenone **1**, a novel flavonoid compound which has an unusual nonaromatic B-ring, was isolated from torres's ferns, such as

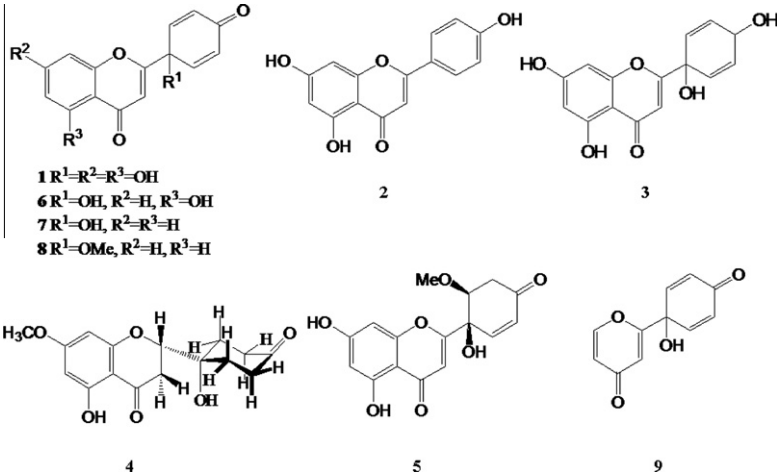

Figure 1. Chemical structures of **1** and related flavonoids.

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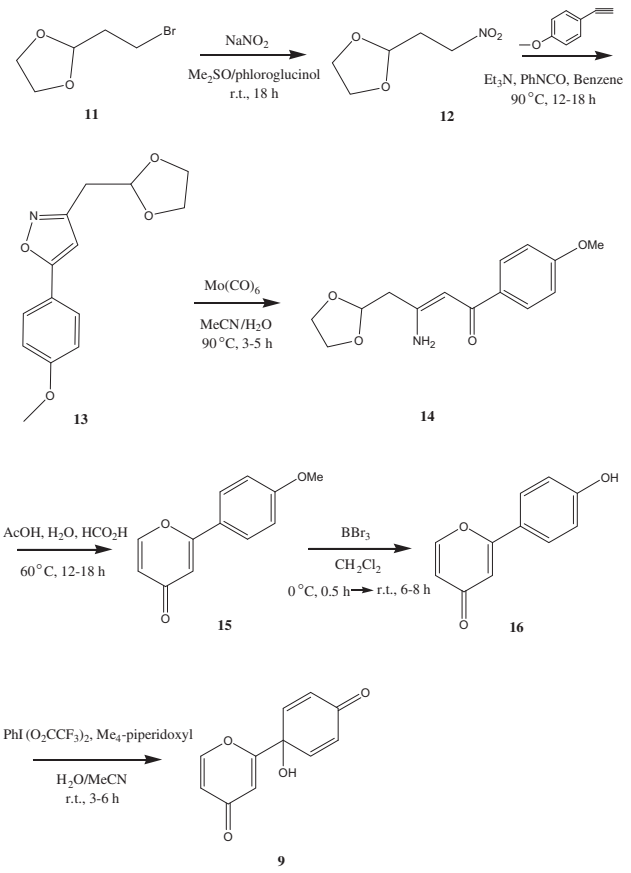

Figure 2. Synthesis of 2-(1-hydroxy-4-oxo-cyclohexa-2,5-dienyl)-pyran-4-one **9**.

Thelypteris torresiana[5] and *Macrothelypteris torresiana*.[6] This kind of flavonoid displayed potent antitumor activity against a broad spectrum of human cancer cell lines such as HepG2 and HepG3 (liver), MDA-MB-231 and MCF-7 (breast), MDAH-2774 and SKOV3 (ovary), LNCap (prostate) and A549 (lung) with IC_{50} values of 0.94–5.59 μM.[4,5,7] Apigenin **2**, however, which is the conceivable biosynthetic precursor of 1 bearing a 4′-hydroxyphenyl B-ring, showed no significant antiproliferative activity.[8] Thus, the promising compound **1** containing the novel nonaromatic B-ring system has attracted considerable synthetic interest and several structure–activity relationship (SAR) studies related to **1** have been reported.[9]

Limited structure–activity correlations of **1** as well as other natural and synthetic analogues (Fig. 1) have defined some of the structural requirements of the cytotoxic pharmacophore. Comparison of **1** to its analogues **2** and **3** showed that the latter two compounds, without oxidation of 4′-hydroxyphenyl, had reduced activity compared with **1**.[8] In addition, compound **5**[9], which has only a single double bond in the B-ring exhibited in general 2–5 magnitudes less active than compound **1** and cyclohexanone **4**[10] was devoid of biological activity. The results confirmed that a symmetrical double bond structure in the B-ring was crucial for potent cytotoxicity.[9] On the other hand, 7-hydrogenated **6** and 5,7-dihydrogenated **7** showed enhanced activity than **1**, indicating that the C-5 and C-7 hydroxyl substituents were not requirements of the pharmacophore. Lastly, 1′-methoxy **8** showed less potent compared with 1′-hydroxy analogues **1**, 6, and **7**, which indicate the hydroxyl substituent at the C-1′ position are essential for recognition.[9] From these results we determined that the 1′-hydroxycyclohexa-2′,5′-dien-4′-one B-ring is required in conjunction with either of the C-ring **9** or the AC-ring **7**. At present, compound **7** is the structurally simplest analogue known that exhibits potent cytotoxicity. Compound **9** may therefore be considered complementary analogues to the presently known minimum pharmacophore in **7**. In this Letter, we report the synthesis and biological evaluation of **9**.

The synthesis of **9** is outline in Figure 2. Nitration reaction of 2-(2-bromoethyl)-1,3-dioxolane 11 with nitrite provided the 2-(2-nitroethyl)-1,3-dioxolane **12**[11] in 50% yield. Under Mukaiyama's dehydration conditions, the nitrile oxide intermediate was generated with phenyl isocyanate from **12**.[12] 1,3-dipolar cycloaddition reaction of nitrile oxide with acetylene to give the isoxazole **13** in 45% yield.[13] Reductive cleavage reaction of the isoxazole was achieved using $Mo(CO)_6$, with the resulting enamino ketone **14** (73% yield) being cyclized to the pyran-4-one derivative **15** in 55% yield under acidic conditions.[13] Demethylation with BBr_3 gave the 2-(4-hydroxyphenyl)pyrone **16** (76% yield), which was oxidized using [bis(trifluoroacetoxy)iodo]benzene (PIFA) to give the novel 2-(1-hydroxy-4-oxo-cyclohexa-2,5-dienyl)-pyran-4-one 9 (22% yield).[16]

Compound **1** and **9** were screened in vitro against eight cancer cell lines, including MCF-7 and MDA-MB-231 (breast cancer), SKOV3 (ovarian cancer), PC-3 (prostate cancer), HepG2 (liver cancer), PANC-1 (pancreatic cancer), RPMI-8226 and U266 (multiple myeloma). The results are graphically represented in Figure 3. The synthetic **9** showed enhanced cytotoxic activity than natural product **1** with notable IC_{50} values of 0.38–3.43 μM against most cancer cells tested (MCF-7, MDA-MB-231, SKOV3, PC-3, HepG2, and PANC-1). In comparison with the presently structurally simplest analogue **7**, compound **9** showed about 2- to 5-fold greater cytotoxicity against MCF-7, MDA-MB-231, and HepG2 cancer cell lines.[9]

The toxicity of **1** and **9** against normal cells were also investigated using human peripheral blood mononuclear cell (PBMC) and human embryonic kidney 293 cell line (HEK 293). The results are shown in Figure 4. Compound **9** was only weakly active against the two normal cells and exhibited selective anticancer activities compared with **1**.

The effectiveness of **1** and **9** were tested in vivo against the hepatoma H22 tumor bearing mice.[14,15] Cyclophosphamide (CTX) was used as reference drug. Compound **9** and CTX at the dosage of 10 mg/kg significantly inhibited the growth of implanted H22 tumor in mice by 34.12% and 41.74%, respectively, while compound **1** was found to be inactive with inhibition rate less than 5%. In addition, after intraperitoneal injection of **1** and **9**, the white blood cell (WBC), thymus index, spleen index and the body weight had no significant difference between control and drug-treated group. In bone marrow cells, however, the numbers of total nucleated cells as well as the ratio of the granulocytic and erythroid cell systems were declined by 51.52% and 36.9%, respectively after treated with **1**, suggesting its damage on bone marrow hematopoiesis. By contrast, no significant difference was observed in the **9**-treated group.

The present study help further define the minimum cytotoxic pharmacophore shared by protoapigenone **1**. The newly synthesized analogue **9** showed better in vitro and in vivo activity and less major side effects compared with **1**, suggesting that the A-ring of the bioflavonoid **1** is not the requirements of the

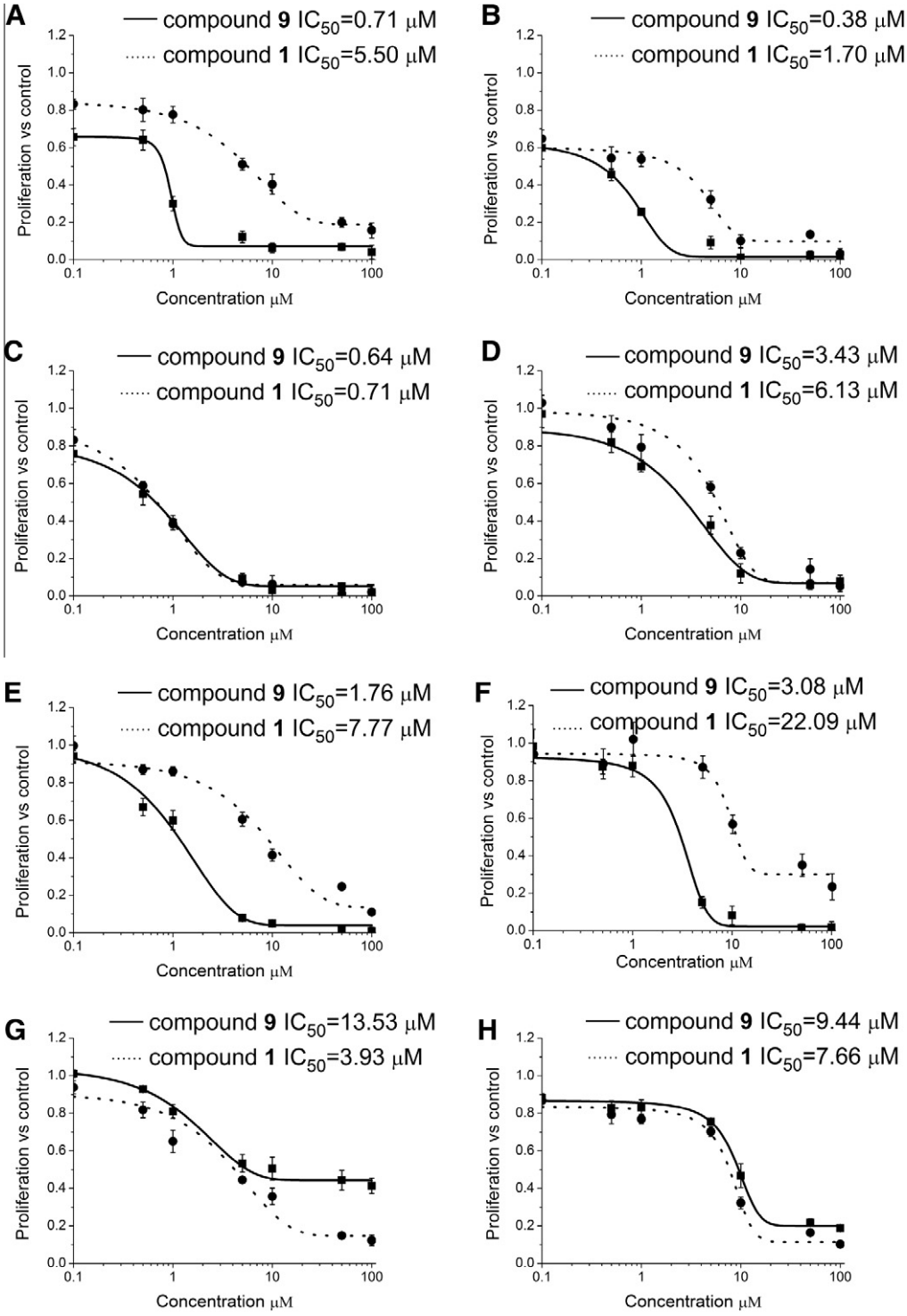

Figure 3. Antitumor activity of compound **1** and **9** in vitro against (A) MCF-7 (breast), (B) MDA-MB-231 (breast), (C) SKOV3 (ovarian), (D) PC-3 (prostate), (E) HepG2 (liver), (F) PANC-1 (pancreas), (G) RPMI-8226 (blood), and (H) U266 (blood) cell lines for 24 h. The cell proliferation rates were determined by MTT assay and they are presented as mean ± SD (n = 4). Cells treated with DMSO (equivalent volume) were used as a vehicle control.

pharmacophore. We tentatively conclude that the minimum structural pharmacophore of **1** must reside in either or both of the B-ring and C-ring. The synthesis of complementary analogues is currently under investigation in our laboratories.

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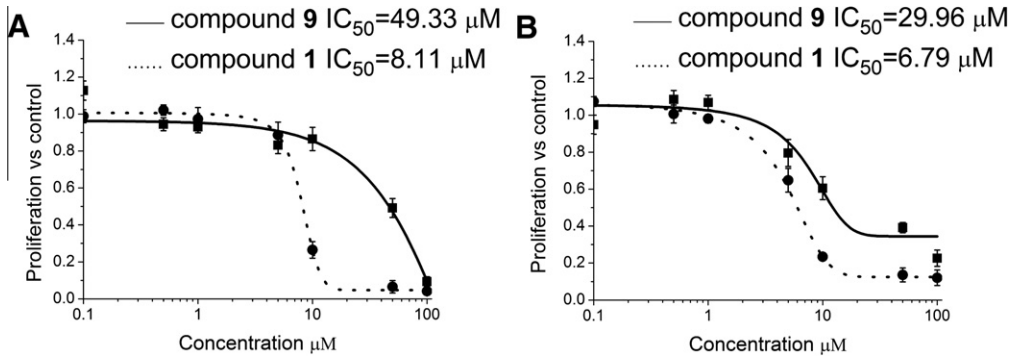

Figure 4. Effect of compound **1** and **9** on human peripheral blood mononuclear cell (PBMC, **A**) and human embryonic kidney 293 cell line (HEK 293, **B**) for 24 h. The cell proliferation rates were determined by MTT assay and they are presented as mean ± SD (n = 4). Cells treated with DMSO (equivalent volume) were used as a vehicle control.

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16. *Experimental*: physico-chemical properties of the synthetic compounds are as follows:
    2-(4-Methoxyphenyl)-4H-pyran-4-one (**15**): ^{1}H NMR (400 MHz, in DMSO): δ 8.15 (1H, d, J = 5.6 Hz, H-6), δ 7.85 (2H, d, J = 8.8 Hz, H-2',6'), δ 7.08 (2H, d, J = 8.8 Hz, H-3',5'), δ 6.81 (1H, d, J = 2.4 Hz, H-3), δ 6.41 (1H, dd, J = 5.6, 2.4 Hz, H-5), δ 3.88 (3H, s, methoxy-CH_3).
    2-(4-Hydroxyphenyl)-4H-pyran-4-one (**16**): ^{1}H NMR (400 MHz, in DMSO): δ 8.17 (1H, d, J = 5.6 Hz, H-6), δ 7.74 (2H, d, J = 8.8 Hz, H-2',6'), δ 6.89 (2H, d, J = 8.8 Hz, H-3',5'), δ 6.75 (1H, d, J = 2.4 Hz, H-3), δ 6.28 (1H, dd, J = 5.6, 2.4 Hz, H-5).
    2-(1-Hydroxy-4-oxo-cyclohexa-2,5-dienyl)-pyran-4-one (**9**): yellow solid; UV λ_{max}(MeOH) 252.5 nm; ^{1}H NMR (400 MHz, in DMSO): δ 8.10 (1H, d, J = 6.0 Hz, H-6), δ 6.96 (2H, d, J = 10.0 Hz, H-2',6'), δ 6.30 (2H, d, J = 10.0 Hz, H-3',5'), δ 6.54 (1H, d, J = 2.4 Hz, H-3), δ 6.29 (1H, dd, J = 6.0, 2.4 Hz, H-5); ^{13}C NMR (100 MHz, in DMSO): δ 185.3 (C-4'), δ 178.2 (C-4), δ 167.1 (C-2), δ 156.9 (C-6), δ 148.4 (C-2',6'), δ 129.1 (C-3',5'), δ 116.9 (C-3), δ 113.5 (C-5), δ 69.0 (C-1'); ESI–MS (m/z): 203 [M$^-$-H, 100]; HRMS (FAB) calcd for $C_{11}H_8O_4$: m/z 204.0422; found: 204.0425.

Biological screening procedure: measurement of cell proliferation was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, 2000 cells were plated out into each well of a 96-well plate and allowed to adhere. Compound **1** and **9** dissolved in dimethylsulfoxide (DMSO, Sigma) and diluted with tissue culture medium, were then added at increasing concentrations (0.1–100 μM, eight wells per concentration). The cells were incubated in the presence of drugs for 24 h. MTT reagent (Sigma) dissolved in phosphate buffered saline was added to each well at a concentration of 0.5 mg/mL, and the cells were incubated for four additional hours. Following this time, the medium containing the MTT reagent was aspirated, and DMSO (150 μL) was added to each well. The absorbance of each well was measured in a microplate reader (Power- Wave XS, Bio-Tek) at a wavelength of 570 nm. The IC_{50} value for each compound tested was determined by plotting concentration versus percent absorbance obtained in the MTT assay.

In vivo effects of **1** and **9** were studied in hepatoma H22 tumor-bearing mice. ICR mice (18–22 weeks) were purchased from Animal Center, Huazhong University of science and technology. The animals were maintained in a standard environmental condition and fed with rodent diet and water ad libitum. All experiments were approved by the institutional Animal Care and Use Committee of Huazhong University of Science and technology. Ascites was taken from hepatoma H22 mice passing on from generation to generation for 6–7 days under aseptic condition, which was diluted with normal saline into a suspended solution in a concentration of 1×10^7 cells/ml. A volume of 0.2 ml of H22 tumor cell suspension was hypodermically injected into the right flank of each mouse. Twenty-four hours after injection of the H22 cells, the mice were randomly divided into four groups, that is, a model group, a compound **1** group, a compound **9** group, a Cyclophosphamide (CTX) group, 10 mice in each group, and with a normal group set as the controls (0.2 ml saline was injected into the right axillary fossa for each mouse of this group). From the second day after inoculation, **1** and **9** dissolved in 10% ethanol solution were administered for seven successive days by intraperitoneal injection (10 ml/kg for each mouse) at daily dose of 10 mg/kg. CTX (10 mg/kg) was used as reference drug. Normal animals and untreated tumor bearing animals were treated with the same volume of 10% ethanol solution. The mice were killed at day nine. The tumors, thymus, and spleen were separated and weighed, the blood sample was taken for count of WBC and the femurs were dissected and marrow cells flushed from the bone. The tumor inhibition rate was calculated: (the tumor weight of the model group—the tumor weight of the treatment group)/the tumor weight of the model group × 100% and the thymus index and the spleen index were calculated: The thymus index = the thymus weight (mg)/body weight (g); while the spleen index = the spleen weight (mg)/body weight (g). Numbers of nucleated cells of the bone marrow were determined using a Coulter Counter. The counts of granulocytic or erythroid cells per femur were performed on marrow smears stained with the May-Grünwald-Giemsa method.