Full Paper

Design and Synthesis of a Gossypol Derivative with Improved Antitumor Activities

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A novel chemical process has been devised for the synthesis of a new derivative of gossypol, 6,7,6′,7′-tetrahydroxy-5,5′-diisopropyl-3,3′-dimethyl-[2,2']binaphthalenyl-1,4,1′,4′-tetraone (Apogossypolone). This new process has only four steps, with a shorter synthesis span, a simple purification process, and improved yield and quality. The structure of apogossypolone was characterized by ^{1}H-nuclear magnetic resonance, ^{13}C-nuclear magnetic resonance, mass spectroscopy, infrared spectroscopy, and elemental analysis. Cell-cytotoxicity assay demonstrates that apogossypolone is three- to six-fold more potent than the parent compound, (−)-gossypol, in inhibiting the human prostate tumor cell lines PC-3 and DU-145 as well as the human breast cancer cell line MDA-MB-231. The colony-formation assay with DU-145 cells showed that apogossypolone inhibited more than 70% of colony formation at 1 μM, whereas (−)-gossypol at 10 μM only inhibited less than 50% of colony formation. The results indicate that apogossypolone exerts strong antitumor activities in human prostate and breast cancer cells, and thus represents a promising cancer therapeutic.

Keywords: Antitumor activity / Apogossypolone / Assay / Gossypol / Synthesis

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Introduction

Gossypol is a yellow polyphenolic compound found in pigment glands distributed throughout the cotton plant (*Gossypium* sp.). It has been associated with a wide range of biological and medicinal activities, including antifertility [1], antimalarial [2], antitumor [3], and antiviral effects [4]. Therefore, it is widely used in the medical and pharmaceutical field. Recently, a number of research reports suggested that gossypol is a promising novel anticancer drug, especially (−)-gossypol, which has received considerable attention because of its unique antimalignancy characteristics [5–7]. Its anticancer activities have been attributed to its binding affinity to Bcl-2, Bcl-xL, and Mcl-1 proteins-rational therapeutic targets because of their roles in regulating the pathway which leads to apoptosis of tumor cells [8–12]. However, it is not widely used clinically because it is very unstable, toxic, and insoluble.

Thus, extensive attempts to chemically modify gossypol to obtain therapeutic antitumor agents with greater potency and less toxicity have been performed. More than 50 new analogues of gossypol have been synthesized to date [13–16]. Unfortunately, none of them has been used in the clinic as an antitumor agent because of undesirable side effects, insolubility and a lack of selectivity against tumor cells. Therefore, (−)-gossypol is still the world's only orally available small-molecule inhibitor of Bcl-2, Bcl-xL and Mcl-1 proteins that has advanced into clinical trials [17].

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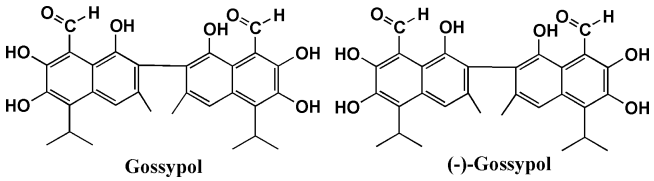


Figure 1. Structures of (–)-gossypol ((–)-G) and gossypol.

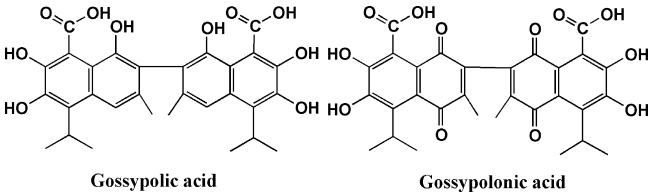


Figure 2. Structures of gossypolic acid and gossypolonic acid.

Although (−)-gossypol represents a potentially interesting lead compound, it can be further optimized for better efficacy of therapy and reduced toxicity as a new drug targeting Bcl-2, Bcl-xL and Mcl-1 proteins [18, 19]. Both (−)-gossypol ((−)-G) and gossypol contain two reactive aldehyde groups in their structures (Fig. 1). These two reactive groups can combine with lysine residues of proteins to form Schiff's bases, which have been assumed to cause the toxicity of gossypol in animals and humans. This toxicity, albeit mild, greatly limits the maximum dose of (−)-gossypol that can be given to patients. In addition, the compound can combine with other proteins when it enters the bodies of animals and humans, causing reduction of therapeutic efficacy and enhancement of toxicity. In cancer clinical trials, gossypol could not be administrated i.v. and has a maximum tolerated dose (MTD) of 40 mg daily when given orally [20]. It is expected that removal of the aldehyde groups will significantly reduce gossypol's toxicity in humans. Moreover, although the binding affinity of (−)-gossypol to Bcl-2 is fairly high (K_i = 237 nM) and it has been one of the first Bcl-2 inhibitors reported, its binding affinity to Bcl-2 protein is significantly less than that of Bad 25-mer BH3 peptide (K_i = 6.9 nM) in binding assay [21–24]. Therefore, despite the fact that (-)-gossypol represents a potentially interesting lead compound, it is necessary to find novel and safer derivatives of gossypol with greatly improved binding affinity to Bcl-2 protein, provided that these new analogues have comparable or better cellular activity in cancer cells with high levels of endogenous Bcl-2 protein, as well as good selectivity in normal and cancer cells with low levels of Bcl-2 protein.

Based upon the mechanism of (−)-gossypol's binding affinity to Bcl-2/Bcl-xL, it has been proposed that one aldyhyde group interacts with a conserved Arginine residue in Bcl-2/Bcl-xL, while the other aldehyde group is exposed to solvent [25–27]. To maintain this interaction with Arg residue in Bcl-2/Bcl-xL, we designed and synthesized two new analogues, gossypolic acid and gossypolonic acid (Fig. 2). These two compounds were determined to have K_i values of 90 nM and 150 nM, respectively, and thus were slightly more potent than (−)-gossypol. However, the two acid groups in these compounds are negatively charged at physiological conditions (pH = 7.4), and this may prevent them from entering cells. Both compounds, in fact, were found, in spite of their excellent binding affinities to Bcl-2, to be poor inhibitors of cell growth in PC-3 cells with IC_{50} values greater than 10 μM.

To overcome the poor cell permeability of gossypolic and gossypolonic acid, we designed and synthesized apogossypolone, in which the two aldehyde groups of gossypol are completely removed. Computational docking of these compounds, followed by lengthy molecular dynamic simulation, revealed that the three hydroxyl groups on the left naphthyl ring in apogossypolone form several highly optimized hydrogen bonds with Arg 139 in Bcl-2, compensating for the removal of the aldehyde group in (−)-gossypol [28, 29]. Apogossypolone was determined to have K_i values of 35 nM. The binding curve for apogossypolone to Bcl-2 is shown in Fig. 3. The K_i value for apogossypolone to Bcl-xL was determined to be 660 ± 40 nM, similar to that of (−)-gossypol. Hence, apogossypolone represents a new and more potent small molecular inhibitor of Bcl-2/Bcl-xL.

In view of the available studies on the structure-activity relationship and in continuation of our ongoing efforts on the design and development of structurally modified gossypol, here, we describe a novel chemical synthesis process of 6,7,6′,7′-tetrahydroxy-5,5′-diisopropyl-3,3'-dimethyl-[2,2′] binaphthalenyl-1,4,1′,4′-tetraone (Apogossypolone) with improved yield, and quality of its antitumor activities.

Results and discussion

Chemistry

We have adopted a concise synthetic route for the preparation of apogossypolone. The target compound **5** was synthesized as shown in Scheme 1. Generally, the two aldehyde groups of gossypol may be removed through heating in a basic solvent. In addition, gossypol may also be decarbonylated by reaction with $HSCH_2CH_2SH$ in the presence of BF_3 / Et_2O. Here, gossypol was decarbonylated by heating in concentrated aqueous sodium hydroxide using a steam bath. The whole reaction process was not

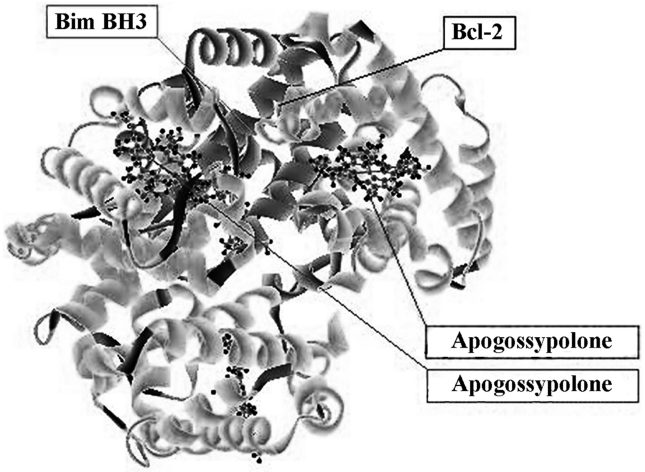
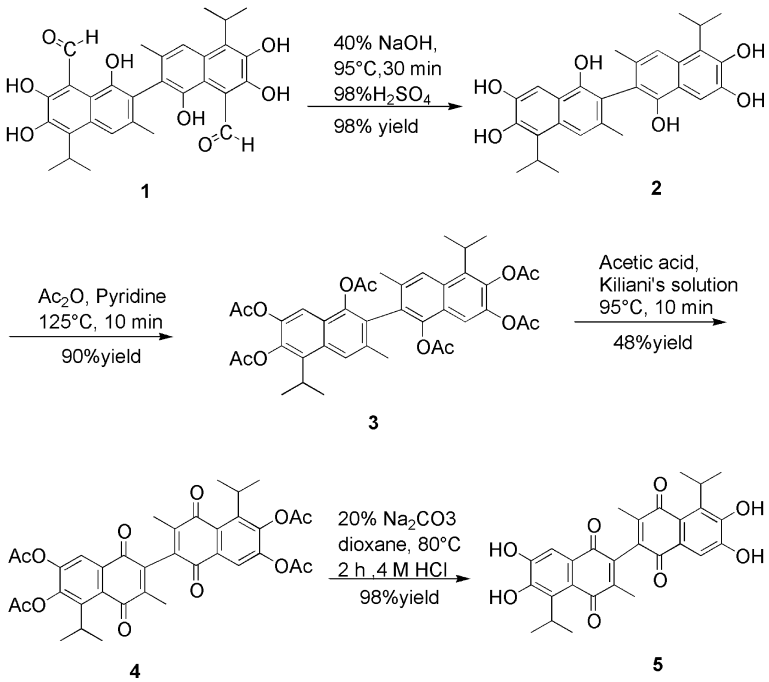


Figure 3. Binding curve for apogossypolone to Bcl-2.

only easy to operate but also lasted only 30 minutes because of the use of the NaOH or KOH instead of other bases. Then, the reaction mixture was poured onto ice containing concentrated sulfuric acid. The resulting precipitate was extracted with ether and further purified through hot methanol and water, first obtaining compound **2**. After optimization, the yield was significantly increased from 90% to 98% by canceling the purification process without affecting the last result. After compound **2** was obtained, we looked for a suitable protection group to shield the hydroxy group in compound **2**; this is important because of its instability. There were many chemical groups we could select, such as alkanoyl, aralkanoyl, benzoyl, methyl, and alkyl groups. Here, we selected the alkanoyl group to protect compound **2** by treatment with anhydride in pyridine at high temperatures. After adding the same equivalent of water, we easily obtained the crude product ester **3** easily. It is noteworthy that the crude compound was purified only by treating it with an equivalent volume of boiling ethyl acetate and petroleum ether, resulting in a 98% yield of pure ester **3**. In addition to these steps, it was important to select a suitable solvent because it directly affected the reaction condition and the yield. Fortunately, we obtained compound **3** by selecting a suitable reagent anhydride and the solvent pyridine, thereby having to heat it for only several minutes at the defined temperatures throughout the whole process.

With the ester **3** in hand, the selection of an oxidation agent was critical in order to get compound **4**. Based on the structure of compound **3**, a powerful oxidation reagent was needed in order to successfully complete the structure modification. With our knowledge of the oxidation reagents, the use of periodic acid with chromium oxide was obvious for its superior oxidation character. However, at last we selected the Kiliani's solution which is a mixture of H_2SO_4, H_2O, Cr_2O_3 in defined proportions. We subjected the ester **3** to seven equivalents Kiliani's sol-



Scheme 1. Synthesis of the target compound **5**.

Table 1. Structures and physico-chemical data of compounds **2**–**5**.

Compound	M.p. (°C)	Reaction time (min)	Yield (%)	Formula	M.W.
2	215–217	30	98	$C_{28}H_{30}O_6$	462.52
3	289–291	10	90	$C_{40}H_{42}O_{12}$	714.74
4	228–230	10	48	$C_{36}H_{34}O_{12}$	658.63
5	217–219	120	98	$C_{28}H_{26}O_8$	490.49

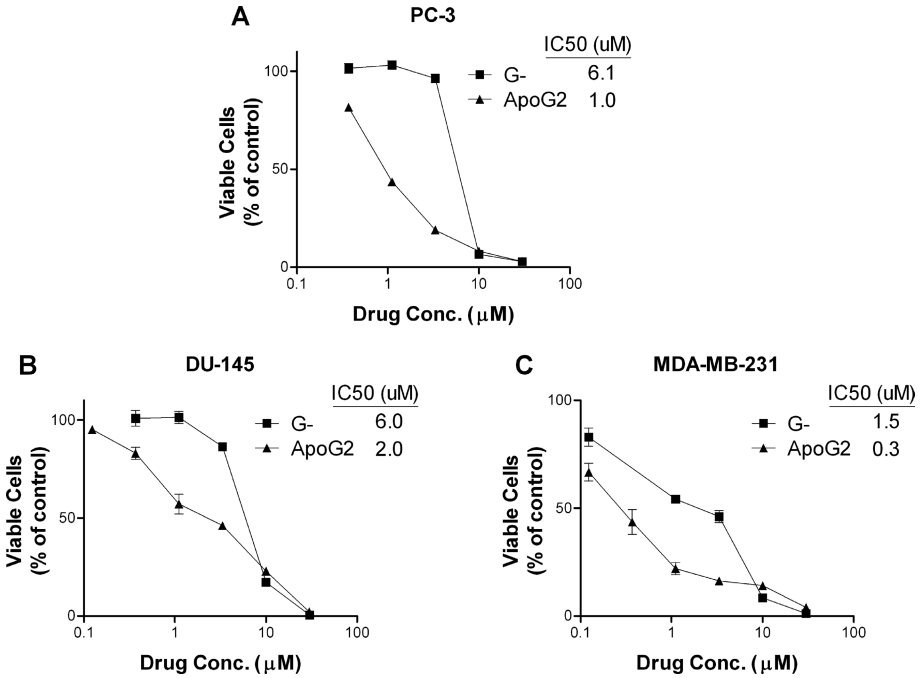


Figure 4. MTT-assay results for compound **5**.

ution at 95°C in acetic acid for only five minutes, followed by addition of 50 equivalents of water, obtaining the crude product ester **4**. The resulting crude product was further purified from hot methanol aided by ultrasound to reach crystallization. The target compound **5** was obtained by removal of the protection groups of ester **4** by reacting it with ten equivalents sodium carbonate in dioxane at 80°C for 2 h. The product was then acidified and isolated by extraction with ether. After purification from the hot methanol and water with the aid of ultrasound. Compound **5** was obtained with a significant yield of 98% (Table 1). As can be seen from the whole synthesis process, we adopted the crystallization and recrystallization to purify the crude product in contrast to using flash silica gel column chromatograpy. In addition, the product yield was also significantly improved by adopting ultrasound crystallization. The structures of the compounds in Scheme 1 were confirmed by infrared spectroscopy (IR), ^{1}H-NMR, ^{13}C-NMR, mass spectroscopy, and elemental analysis.

2.2 Biological evaluation

To test the anticancer activity of compound **5** obtained with the new synthesis method, the MTT-based cell cytotoxicity assay and colony-formation assay were carried out. Figure 4 shows the MTT assay results. Compound **5** has IC_{50} values 1.0, 2.0, and 0.3 μM, three- to sixfold more potent than (−)-gossypol which has IC_{50} of 6.1, 6.0, and 1.5 μM in human prostate cancer cell lines, PC-3 and DU-145, and human breast cancer cell line MDA-MB-231, respectively. The colony formation assay was conducted in DU-145 cells. Figure 5 shows that apogossypolone potently inhibited the colony formation of DU-145 cells. At 1 μM, apogossypolone inhibited more than 70% of colony formation compared with that of the solvent control, whereas (−)-gossypol, at 10 μM, only inhibited less than 50% of colony formation. These data suggest that the apogossypolone we synthesized has strong antitumor activity. Regarding the antitumor mechanism of apogossypolone, the likely mechanism is that apogossypolone binds to Bcl-2 (or Mcl-1, Bcl-xL, A1, Bcl-w) and prevents its associ-

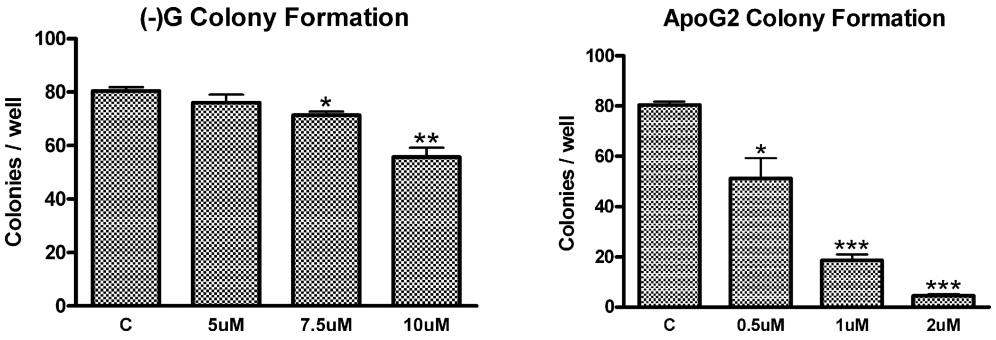


Figure 5. Inhibition of the colony formation of DU-145 cells by apogossypolone.

ation with BH3-only pro-apoptotic proteins, thus unleashing the pro-apoptotic proteins to participate in the apoptotic response. Yet, it should be noted that the exact mechanism of action of apogossypolone is unclear to date. Currently, apogossypolone has been reported to be a strong inhibitor of Bcl-2, Bcl-xL and Mcl-1 proteins both *in vitro* and *in vivo* according to our earlier report [31], as well as in a follicular small cleaved cell lymphoma model, nasopharyngeal carcinoma xenografts, and human leukemia in a recent study [30, 33]. Our experiment further proved that apogossypolone has antitumor activity in the prostate cancer model.

Apogossypolone showed potent radiosensitization potential in the prostate cancer PC-3 xenograft model in nude mice via oral administration, leading to complete tumor regression in seven out of ten tumors in the combination treatment group [31]. Besides these results, apogossypolone is very stable with no decomposition detected when it was stored at room temperature for several weeks without the protection of nitrogen. Because the major toxicity of gossypol in animals and humans has been associated with its two reactive aldehyde groups, removal of these aldehydes should significantly reduce the toxicity. Our earlier studies have examined the maximal tolerated dose (MTD) of apogossypolone and (-)-gossypol using two different routes of administrations oral and i.v. Via both routes of administration, the MTD of apogossypolone was 240 mg/kg (orally) and 80 mg/kg (i.v.), eight times better than (−)-gossypol, with MTD of 30 mg/kg and 10 mg/kg, respectively [31]. All the results indicated that apogossypolone was superior to (−)-gossypol, possibly making apogossypolone the most potent gossypol derivative identified to date.

Conclusions

In conclusion, we have designed a novel synthesis method for apogossypolone with only four steps, with a shorter synthesis span, lower cost, a simple purification process, and improved quality. Our results differ from another recent report [32]. In addition, it is noteworthy that we applied ultrasound crystallization, which improved the yield significantly. The MTT-based cell-cytotoxicity assay and colony-formation assay show that apogossypolone has significantly improved antitumor activity in the prostate-cancer model compared to the parent compound (-)-gossypol. Since the anticancer mechanism is not clear, further research in this area is in progress in our laboratories.

Experimental

Synthesis

All reagents were purchased from commercial sources and were used without further treatment, unless otherwise indicated. Melting points were determined with a Mettler FP82 + FP80 apparatus (Mettler-Toledo, Greifensee, Switzerland) and have not been corrected. The ^{1}H-NMR and ^{13}C-NMR spectroscopy were recorded on a Bruker 400 Ultrashield™ (Bruker, Rheinstetten, Germany), using TMS as the internal standard. IR spectroscopy were obtained using a ThermoNicolet FT-IR Nexus spectrophotometer (Thermo Electron Corporation, Waltham, MA, USA) with samples as KBr pellets. Elemental microanalyses were carried out on vacuum-dried samples using an elemental analyzer (LECO, CHN-900 Elemental Analyzer; LECO Corporation, St. Joseph, MI, USA) and were in an acceptable range of ± 0.4% for all compounds. Mass spectra were recorded using a Hewlett-Packard MSD 5973N spectrometer (GC 6890plus/DIP; Agilent Technologies, Inc., Santa Clara, CA, USA).

(+)-5,5′-Diisopropyl-3,3′-dimethyl-[2,2′]binaphthalenyl-1,6,7,1′,6′,7′-hexaol ***2***

Gossypol acetic acid (5.0 g, 8.7 mmol) was heated in 40% aqueous sodium hydroxide (30 mL) at 95°C for 30 min, then the reaction mixture was poured onto ice-containing 98% concentrated sulfuric acid. The resulting mixture was extracted with ether, the combined extracts were washed with water, and concentrated to yield crude **2**. Recrystallization from methanol and water afforded pure product **2** (4.9 g, 98%). Brown solide; m.p.: 215–217°C. ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 1.54 (d, *J* = 6.56 Hz, 12H, 12-CH_3, 12′-CH_3, 13-CH_3, 13′-CH_3), 2.14 (s, 6H, 14-CH_3, 14′-CH_3), 3.52 (m, 2H, 11-CH), 5.0 (s, 6H, 1-C-OH, 1′-C-OH, 6-C-OH, 6′-C -OH, 7-C-

OH, 7′-C-OH), 7.26 (s, 2H, 4-C-H, 4′-C-H); ^{13}C-NMR (75 MHz, MeOD) δ: 147.5, 116.9, 130.6, 113.1, 146.7, 145.7, 100.2, 118.8, 127.0, 18.0, 25.2, 16.8. IR (KBr, cm^{-1}): 3427, 2872, 1615, 1594,1269. Anal. Calcd. (%) for $C_{28}H_{30}O_6$: C, 72.65; H, 6.49. Found: C, 72.64; H, 6.50.

Acetic acid 1,7,1′,6′,7′-pentaacetoxy-5,5′-diisopropyl-3,3′-dimethyl-[2,2′]binaphthalenyl-6-yl ester ***3***

Acetic anhydride (9 mL, 96.3 mmol) was added to the solution of compound **2** in pyridine (20 mL). The reaction mixture was heated at 125°C for 10 min and then allowed to cool and stand at room temperature for 30 min. Water (250 mL) was added to the reaction mixture and crystals of compound **3** formed. Recrystallization from boiling ethyl acetate and petroleum ether afforded pure product **3** (4.25 g, 90%). Pale yellow solid; m.p.: 289-291°C. ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 1.49 (d, *J* = 6.56 Hz, 12H, 12-CH_3, 12′-CH_3, 13-CH_3, 13′-CH_3), 2.31-2.39 (m, 6H, 14-CH_3, 14′-CH_3), 3.82 (s, 2H, 11-CH), 7.98 (s, 2H, 4-C-H, 4′-C-H), 7.26 (s, 2H, 8-C-H, 8′-C-H), 2.04 (s, 16-CH_3, 16′-CH_3, 18-CH_3, 18′-CH_3, 20-CH_3, 20′-CH_3); ^{13}C-NMR (75 MHz, $CDCl_3$) δ: 141.2, 126.9, 135.7, 122.7, 124.5, 144.9, 113.7, 131.1, 168.6, 27.5, 21.6, 20.7. IR (KBr, cm^{-1}): 3444, 2960, 1770, 1592,1608. Anal. Calcd. (%) for $C_{40}H_{42}O_{12}$: C, 67.16; H, 5.88. Found: C, 67.17; H, 5.87.

Acetic acid 6,6′,7′-triacetoxy-5,5′-diisopropyl-3,3′-dimethyl-1,4,1′,4′-tetraoxo-1,4,1′,4′-tetrahydro-[2,2′]binaphthalenyl-7-yl ester ***4***

Kiliani's solution (25 mL) was added to a solution of compound **3** in acetic acid (200 mL) and the reaction mixture was stirred at 95°C for 10 min. Ice water (350 mL) was added to quench the reaction and the yellow amorphous compound **4** was formed. Recrystallization from hot methanol afforded pure compound **4** (1.7 g, 48%). Yellow solid; m.p.: 228-230°C. ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 7.26 (s, 2H, 8-C-H, 8′-C-H), 3.82 (s, 2H, 11-CH), 1.36 (m, 12H, 12-CH_3, 12′-CH_3, 13-CH_3, 13′-CH_3), 1.98-2.02 (m, 6H, 14-CH_3, 14′-CH_3), 2.31 (s, 16-CH_3, 16′-CH_3, 18-CH_3, 18′-CH_3); ^{13}C-NMR (75 MHz, $CDCl_3$) δ: 184.2, 146.7, 144.7, 165.2, 166.7, 120.8, 122.6, 125.0, 18.7, 25.6, 13.1, 179.2, 19.7. IR (KBr, cm^{-1}): 3462, 2960, 1770, 1662, 1373, 1200. Anal. Calcd. (%) for $C_{36}H_{34}O_{12}$: C, 65.59; H, 5.16. Found: C, 65.60; H, 5.17.

6,7,6′,7′-Tetrahydroxy-5,5′-diisopropyl-3,3′-dimethyl-[2,2′]binaphthalenyl-1,4,1′,4′-tetraone ***5***

A 20% solution of sodium carbonate (7 mL) was added to a solution of compound **4** (0.5 g, 0.9 mmol) in dioxane (10 mL) and the reaction mixture was stirred at 80°C for 2 h. After cooling, 4 M HCl was added to the solution and the pH was adjusted to 4. Ether was added, and the aqueous phase was extracted three times with ether. The combined extracts were dried and yielded the crude compound **5**. Recrystallization from methanol and water afforded pure compound **5** (0.45 g, 98%). Brick red solid; m.p.: 217–219°C. ^{1}H-NMR (300 MHz, MeOD) δ: 7.26 (s, 2H, 8-C-H, 8′-C-H), 3.27–3.29 (m, 2H, 11-CH), 1.42 (d, *J* = 6.56 Hz, 12H, 12-CH_3, 12′-CH_3, 13-CH_3, 13′-CH_3), 5.0 (s, 4H, 6-C-OH, 6′-C-OH, 7-C-OH, 7′-C-OH), 7.26 (s, 2H, 8-C-H, 8′-C-H), 1.95 (s, 6H, 14-CH_3, 14′-CH_3); ^{13}C-NMR (75 MHz, MeOD) δ: 185.0, 146.8, 145.7, 148.9, 108.4, 135.2, 17.1, 24.8, 11.6. IR (KBr, cm^{-1}): 3447, 2970, 1770, 1662, 1592. Anal. Calcd. (%) for $C_{28}H_{26}O_8$: C, 68.50; H, 5.30. Found: C, 68.51; H, 5.29. MS (m/z): 489.1 $[M]^-$, 491.0 $[M]^+$.

Antitumor-activity assay

Cell culture and reagents

Prostate cancer cell lines used were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were routinely maintained in an improved minimal essential medium (Biofluids, Rockville, MD, USA) with 10% fetal bovine serum and 2 mM L-glutamine. Cultures were maintained in a humidified incubator at 37°C and 5% CO_2. Apogssypolne was dissolved in DMSO at 20 mM as the stock solution.

Growth / cytotoxicity assay

Cell-growth inhibition by Apogossypolone was determined by the MTT-based cytotoxicity assay using Cell Proliferation Reagent WST-1 (Roche) according to the manufacturer's instruction. Briefly, cancer cells (5000 cells/well) were plated in 96-well culture plates, and various concentrations of apogossypolone were added to the cells in triplicates. Four days later, WST-1 was added to each well and incubated for 1.5 h at 37°C. Absorbance was measured with a plate reader at 450 nm with correction at 650 nm. The results are expressed as the % of absorbance of treated wells versus that of vehicle control. IC_{50}, the drug concentration giving 50% growth inhibition was calculated via sigmoid curve fitting using GraphPad Prism 5.0 (GraphPad, Inc.).

Colony-formation assay

The colony-formation assay was conducted in DU-145 cells. Two hundred cells were plated in each well of a six-well plate, and 24 h later, (−)-gossypol and apogossypolone with appropriate doses were added. After 5 days of incubation, 0.5 mL serum was supplemented to each well. The colonies were stained with crystal violet on day 14 and the colonies with over 50 cells were counted.

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