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Original article

Chiral gossypol derivatives: Evaluation of their anticancer activity and molecular modeling

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ABSTRACT

To better use gossypol to find promising anticancer compounds, a series of new and known bis-Schiff base analogs of chiral gossypol were synthesized, and their anticancer activity on HeLa, U87 and M85 cells was tested. The results showed that through a simple chemical modification, less active (+)-gossypol could be converted into more active derivatives. When compared with (−)-gossypol, many more potent compounds that could be the promising anticancer agents were found, and some of them were more potent than the anticancer drug Cisplatin against all three cancer cell lines. By eliminating target functional groups, we observed that the major contributor to the anticancer activity of chiral gossypol seemed to be the phenolic groups, and not the aldehyde groups. Through comprehensive analysis of chiral gossypol analogs, the structure–activity relationships were elaborated.

1. Introduction

Gossypol, a naturally occurring yellow pigment, that is present in members of the *Malvaceae* family such as the cotton plant *Gossypium* species and the tropical tree *Thespesia populnea* [1]. Because of its toxicity, gossypol was proposed to be part of the plant's defense system against pathogenic fungi and insects, and it was regarded as an unwanted processing component [2]. Gossypol was not considered to be a valuable natural product with useful biological activities until its anticancer and male infertility activities were discovered [3,4]. Recently, gossypol and its analogs have been studied extensively for their broad-spectrum of biological activities, such as anti-parasitic [5,6], anti-malarial [7–9], anti-HIV [10,11] and anticancer [12–14].

In solvent, gossypol exists in equilibrium between aldehyde and lactol tautomers, and the shift of the equilibrium depends on the nucleophilicity of the solvent used [15,16]. Because of restricted rotation around the C_2–$C_{2'}$ internaphthyl bond, gossypol has two optically active forms: the (−) and the (+)-enantiomers, which correspond to (R)-gossypol (M-form) and (S)-gossypol (P-form), respectively (Fig. 1). Studies have suggested that gossypol could inhibit the process of mitosis in human mammary cancer cells [17,18], and (−)-gossypol, which affects cells at a lower concentration, was usually more potent in most biological evaluations in comparison with (+)-gossypol or racemic gossypol [19–21]. (−)-Gossypol is currently being clinically evaluated as an anticancer drug in patients with advanced malignancies due to its high affinity for Bcl-2 and Bcl-xL [22]. More recently, the design and synthesis of gossypol analogs have become an exciting research area. Preclinical studies have suggested some gossypol analogs as new non-peptide small molecule inhibitors of Bcl-2 [23,24].

Derivatives such as gossypol Schiff bases prepared by modifying gossypol's aldehyde groups were supposed to reduce its host toxicity while retaining its therapeutic effects [25,26]. To date, most studies on gossypol derivatives have mainly focused on the biological activity of racemic products or a few (−)-gossypol derivatives. There are few reports about the comprehensive comparison of the cytotoxic potential of chiral gossypol derivatives, especially the (+)-isomers. In contrast to the vast amount of literature pertaining to the condensation reaction between amines and gossypol to obtain all kinds of Schiff bases, only a few reports have been published describing the activity of these compounds. Furthermore, the substituent groups of the Schiff bases in those reports only centered on a few long-chain aliphatic amines and amino acid methyl esters [21,27], and the pivotal roles played by the physicochemical properties of the substituents, such as bulk, sterics and

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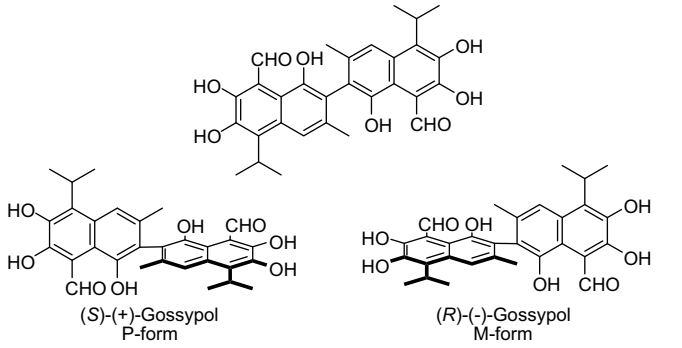


Fig. 1. The chemical and enantiomeric structures of gossypol. The (P)-form, or (*S*)-gossypol enantiomer, corresponds to (+)-gossypol, and the (M)-form, or (*R*)-gossypol enantiomer, corresponds to (−)-gossypol.

hydrophilicity, on their activity could not be discerned. As for structure–activity comparisons, some researchers have pointed out that the cytotoxicity of gossypol was associated with the presence of the aldehyde groups, which could easily bind with biologically important nucleophilic targets. Blocking both aldehyde groups could abolish activity against both pigmented and non-pigmented melanoma cell lines [14], and some have reported that the cytotoxicity of gossypol and its derivatives depends on the availability of the phenolic hydroxyl groups [27]. Others investigated the metabolic fate of gossypol and pointed out that part of its cytotoxicity could be attributed to its transformation into gossypolone, which is the major metabolite of gossypol in vivo [28,29]. However, concerning which functional groups have a decisive effect on the cytotoxicity and the difference in biological properties of the (+) or (−) isomers, no further information has been given.

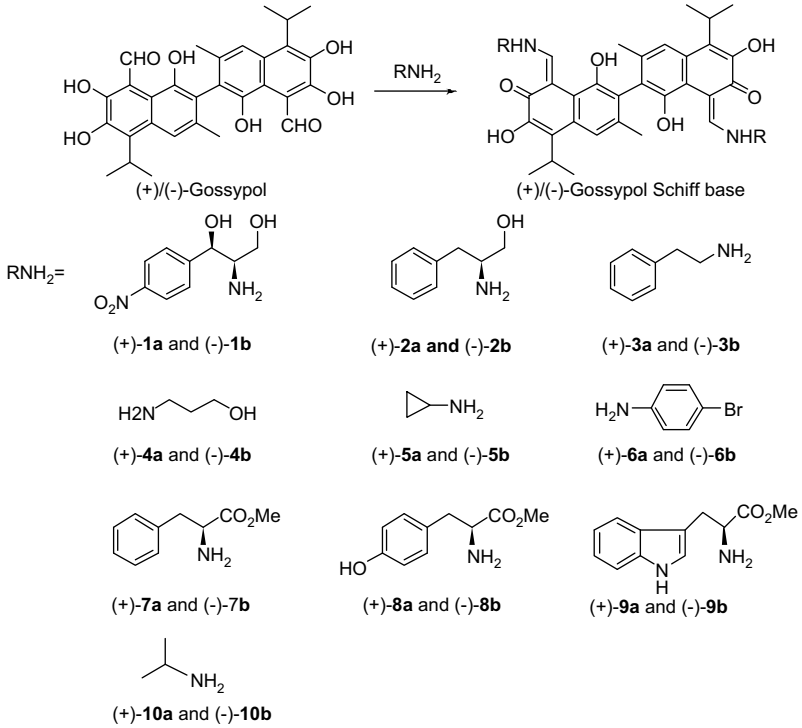
In this study, we focused our attention on the development of new chiral gossypol analogs for the purpose of finding new anticancer agents. During biological evaluation, a very interesting phenomenon attracted us, as some (+)-gossypol Schiff bases showed comparable activity with (−)-gossypol. This prompted us to carry out further comprehensive comparisons of the cytotoxicity of chiral gossypol analogs and their structure–activity relationships to investigate what kind of groups has a decisive effect on the activity. According to this, hydrophilic and hydrophobic amines with markedly different sterics were selected as reactants, and a series of new and known chiral gossypol bis-Schiff bases were synthesized. Their anticancer activities on three representative human tumor cell lines, HeLa (epithelial cervix cancer cells), U87 (brain malignant glioma cells) and M85 (gastric cancer cells), were evaluated. Furthermore, by eliminating target functional groups, a series of chiral gossypol analogs were synthesized and tested to evaluate the influence of target functional groups and their changes on the anticancer activity.

2. Chemistry

A practical procedure has been developed by us for the resolution of racemic gossypol [30]. (−)-Gossypol and (+)-gossypol were simply prepared by acid hydrolysis of the Schiff bases (+)-gossypol bis-(L-tryptophan methyl ester) and (−)-gossypol bis-(L-tryptophan methyl ester) in 90% and 95% yield with ee >95%, respectively. A series of chiral gossypol bis-Schiff bases were prepared by treating (+)-gossypol and (−)-gossypol with the corresponding amine in a suitable solvent to form adduct in high yield (quantitative) (Scheme 1) [31].

3. Results and discussion

The in vitro anticancer activity of gossypol atropisomers and their derivatives on HeLa, U87 and M85 cell lines using an MTT assay was evaluated, and the results are summarized in Table 1. For the sake of comparison, the parent compound (−)-gossypol and anticancer drug Cisplatin were chosen as two reference substances. From these results, we could observe that in each pair, the (−)-derivative was



Scheme 1. Reagents and conditions. For **1** and **2**: methanol, rt; for **3**, **4**: toluene, rt; for **6**: $CHCl_3$, rt; for **7**, **8** and **9**: CH_3OH, NaOH, 55 °C, 4 h; R-NH_2, for **10**: ether, nitrogen, rt.

Table 1
Inhibition of HeLa, U87 and M85 by gossypol atropisomers and their derivatives.

Compound	IC_{50}[a] (μM)			Relative IC_{50}[b] ((−)-gossypol)			Relative IC_{50}[c] (Cisplatin)		
	HeLa	U87	M85	HeLa (17.8)	U87 (30.2)	M85 (18.4)	HeLa (12.6)	U87 (17.1)	M85 (14.9)
(−)-Gossypol	17.8	30.2	18.4	1.0	1.0	1.0	1.41	1.77	1.23
(+)-Gossypol	31.3	59.6	39.7	1.76	1.97	2.16	2.48	3.49	2.66
(+)-**1a**	19.6	30.6	19.0	1.10	1.01	1.03	1.56	1.79	1.28
(−)-**1b**	8.7	13.3	<2.5	0.49	0.44	<0.14	0.69	0.78	<0.17
(+)-**2a**	26.5	29.5	21.5	1.49	0.98	1.17	2.10	1.73	1.44
(−)-**2b**	14.7	29.3	15.5	0.83	0.97	0.84	1.17	1.71	1.04
(+)-**3a**	51.8	>80	>80	2.91	–	–	4.11	–	–
(−)-**3b**	39.9	>80	>80	2.24	–	–	3.17	–	–
(+)-**4a**	10.7	29.0	21.1	0.60	0.96	1.15	0.85	1.70	1.42
(−)-**4b**	12.5	12.1	5.4	0.70	0.40	0.29	0.99	0.71	0.36
(+)-**5a**	55.9	>80	17.4	3.14	–	0.95	4.44	–	1.17
(−)-**5b**	13.5	32.4	17.5	0.76	1.07	0.95	1.07	1.89	1.17
(+)-**6a**	73.7	>80	>80	4.14	–	–	5.85	–	–
(−)-**6b**	49.2	>80	78.4	2.76	–	4.26	3.90	–	5.26
Cisplatin	12.6	17.1	14.9	0.71	0.57	0.81	**1.0**	1.0	1.0

[a] IC_{50} values were the means of three independent experiments run in duplicate.
[b] Relative IC_{50}, compounds' IC_{50} divided by (−)-gossypol's IC_{50}.
[c] Relative IC_{50}, compounds' IC_{50} divided by cisplatin's IC_{50}.

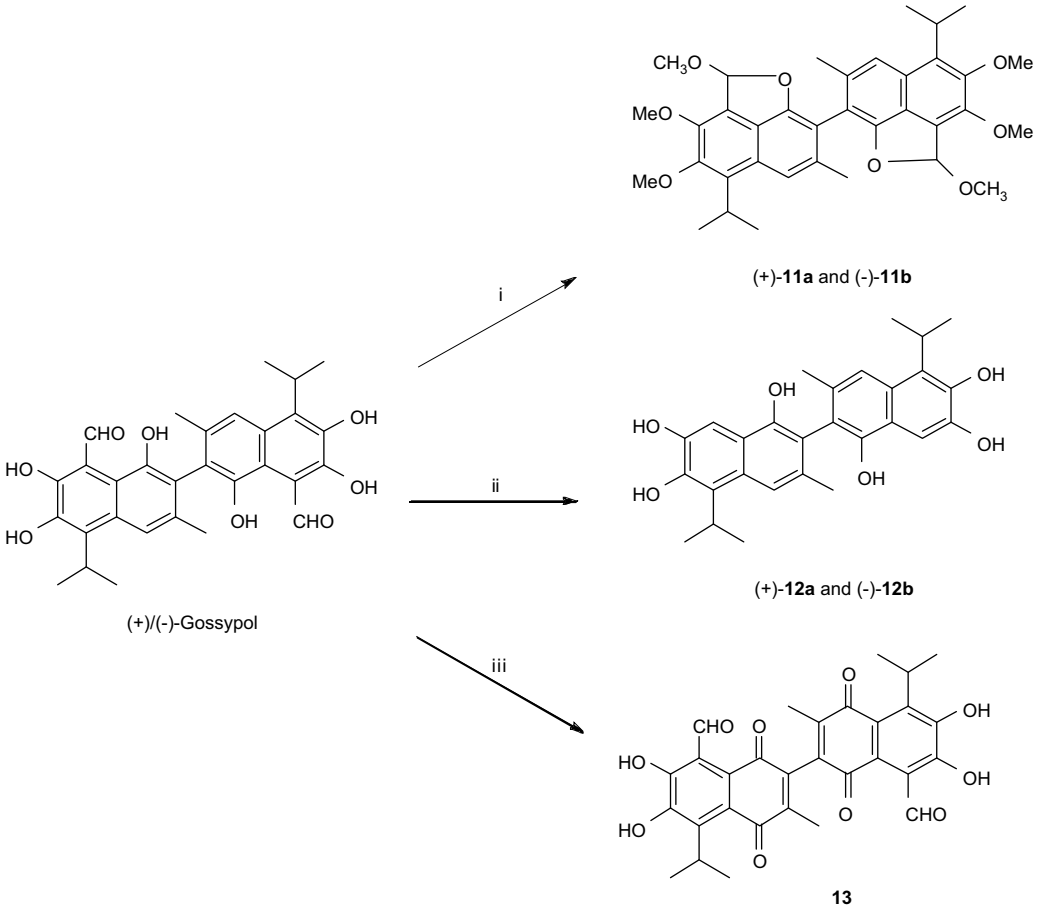
significantly more effective than the corresponding (+)-derivative against all three cancer cell lines, which is consistent with the literature [20,21]. Additionally, **1b** was significantly more cytotoxic against the M85 cell line (IC_{50}, <2.5 μM) and HeLa cell line (IC_{50}, 8.7 μM), while **4b** showed the strongest activity on the U87 cell line (IC_{50}, 12.1 μM). Furthermore, when compared with (−)-gossypol, some modified derivatives, such as **1b**, **2b**, **4a**, **4b** and **5b**, exhibited more powerful activity on the HeLa cell line. **1b**, **2a**, **2b**, **4a** and **4b** were more cytotoxic against the U87 cell line, and **1b**, **2b**, **4b**, **5a** and **5b** were more effective against the M85 cell line. Combined with their structures, **1b**, **2b** and **4b**, with hydrophilic groups substituting the aldehyde groups, showed stronger anticancer activity than (−)-gossypol. **1a**, **2a** and **4a** had similar effects when compared with (−)-gossypol, but remarkably more potent activity in comparison with (+)-gossypol on all three cancer cell lines. Conversely, those compounds with hydrophobic groups substituting the aldehyde groups, such as **3a**, **3b**, **6a** and **6b**, were significantly less cytotoxic against cancer cell lines in comparison with their parent compounds. Based on these data, we could deduce that the hydrophilic group replacing the aldehyde group enhanced the activity against cancer cell lines, while hydrophobic groups may weaken this activity in vitro. This could partially be explained by the fact that compounds with hydrophilic groups substituting the aldehyde more easily couple to the amino acid side chains of proteins or enzymes involved in the cellular growth process [32].

A very interesting exception that might be related to their molecular volume was observed in the case of compounds **5a** and **5b**. With hydrophobic groups substituting the aldehyde groups, the cytotoxic properties of **5a** and **5b** were shown to be quite selective among the cancer cell lines, and their cytotoxicity was especially potent on M85 cells, but weaker on U87 in comparison with (−)-gossypol. Conversely, compounds **3a**, **3b**, **6a** and **6b**, because of the larger bulk of the substituting groups, had remarkably weaker activity on all three cancer cell lines in comparison with **5a** and **5b**. When compared with compounds **2a** and **2b**, we found that more active compounds like **4a** and **4b** also had smaller molecular volume, whereas compounds **1a** and **1b**, with larger bulk but stronger activity, indicated that the hydrophilic property of the substituent groups probably played a more important role than molecular volume in those compounds. Because the substituent groups of gossypol Schiff bases reported previously were very limited and simple, few reports describing the effects of the nature of the substituents on their activity, such as bulk and hydrophilicity, have been published. Recently, the mechanism of cell growth

Table 2
Inhibition of HeLa, U87 and M85 by gossypol atropisomers and their derivatives.

Compound	IC_{50}[a] (μM)			Relative IC_{50}[b] gossypol)			Relative IC_{50}[c] (Cisplatin)		
	HeLa	U87	M85	HeLa (17.8)	U87 (30.2)	M85 (18.4)	HeLa (12.6)	U87 (17.1)	M85 (14.9)
(−)-Gossypol	17.8	30.2	18.4	1.0	1.0	1.0	1.41	1.77	1.23
(+)-Gossypol	31.3	59.6	39.7	1.76	1.97	2.16	2.48	3.49	2.66
(+)-**7a**	>80	>80	>80	–	–	–	–	–	–
(−)-**7b**	69.0	>80	64.4	3.88	–	3.5	5.48	–	4.32
(+)-**8a**	21.8	32.1	52.4	1.22	1.06	2.84	1.73	1.88	3.52
(−)-**8b**	11.4	14.5	16.3	0.64	0.48	0.89	0.90	0.85	1.09
(+)-**9a**	>80	>80	>80	–	–	–	–	–	–
(−)-**9b**	>80	> 80	52.3	–	–	2.84	–	–	3.51
(+)-**10a**	23.8	29.1	34.2	1.34	0.96	1.86	1.88	1.70	2.30
(−)-**10b**	17.1	24.4	30.4	0.96	0.81	1.66	1.36	1.43	2.04
(+)-**11a**	>80	>80	>80	–	–	–	–	–	–
(−)-**11b**	>80	>80	>80	–	–	–	–	–	–
(+)-**12a**	25.5	35.5	>80	1.43	1.17	–	2.02	2.08	–
(−)-**12b**	14.1	28.5	61.2	0.79	0.94	3.33	1.12	1.67	4.11
13	23.3	26.1	28.3	1.31	0.86	1.54	1.85	1.53	1.90
Cisplatin	12.6	17.1	14.9	0.71	0.57	0.81	1.0	1.0	1.0

[a] IC_{50} values were the means of three independent experiments run in duplicate.
[b] Relative IC_{50}, compounds' IC_{50} divided by (−)-gossypol's IC_{50}.
[c] Relative IC_{50}, compounds' IC_{50} divided by cisplatin's IC_{50}.



Scheme 2. Reagents and conditions: (i) Me_2SO_4 methanol, rt 0.5 h, then 4 M aq. KOH, 0.5 h; (ii) 40% aq. NaOH, 90 °C, 3 h, then 10 M aq. H_2SO_4, ether; (iii) $FeCl_3$, acetone, AcOH, 65 °C, 40 min, then ether, 20% aq. H_2SO_4.

inhibition and apoptosis induced by gossypol and some of its analogs was investigated, and these findings suggested that the apoptosis of cancer cells triggered by these compounds was likely to be complex and involve multiple receptors at various sites [22,24,29]. Chiral gossypol Schiff bases with large bulk probably cannot interact adequately with those receptors because of the limited space of the active sites.

For structure–activity comparison and validation, compounds **7a**, **7b**, **8a**, **8b**, **9a**, **9b**, **10a** and **10b** were tested (Table 2). As expected, the results supported our hypothesis. Compounds **8a** and **8b**, with hydrophilic groups substituting the aldehyde, were remarkably more effective against HeLa and U87 cancer cells in comparison with their parent compounds. With hydrophobic groups substituting the aldehyde groups, (+)-gossypol Schiff bases **7a** and **9a** lost their activity completely, and of those derived from (−)-gossypol, only compound **7b** showed very weak activity on HeLa cells (IC_{50}, 69.0 μM) and M85 cells (IC_{50}, 64.4 μM), while compound **9b**, due to its remarkably large molecular bulk, was nearly inactive. Conversely, because of the small volume of the substituting groups, the cytotoxicity of compounds **10a** and **10b**, as compared with their parent compounds, was enhanced on the HeLa and U87 cell lines. These data suggest that gossypol's aldehyde groups might not play a major role in its anticancer activity.

For a comprehensive analysis of gossypol's aldehyde groups, phenolic groups and the substituent groups of the aldehyde, we also synthesized the protected hexamethoxy ethers of gossypol atropisomers **11a** and **11b** and apogossypol atropisomers **12a** and **12b**, (Scheme 2), and investigated their anticancer activity on the

Table 3
Results of the molecular modeling study for gossypol atropisomers and their derivatives.

Compound	Vdw.	Elect.	Total	Dihedral	Distance
(+)-Gossypol	147.431	9.8744	157.305	99.88	8.75
(−)-Gossypol	147.431	9.8744	157.305	−99.88	8.75
1a	168.864	47.1606	216.025	99.83	6.13
1b	168.864	47.1606	216.025	−99.83	6.13
2a	189.720	−10.5496	179.171	107.52	15.08
2b	189.720	−10.5496	179.171	−107.52	15.08
3a	177.842	−7.6072	170.235	84.76	5.93
3b	177.842	−7.6072	170.235	−84.76	5.93
4a	124.942	32.2927	157.235	68.08	4.21
4b	124.942	32.2927	157.235	−68.08	4.21
5a	125.838	−22.8864	102.951	72.19	5.31
5b	125.838	−22.8864	102.951	−72.19	5.31
6a	188.626	−7.7342	180.892	112.56	13.55
6b	188.626	−7.7342	180.892	−112.56	13.55
7a	179.189	52.1285	231.318	62.21	6.54
7b	179.189	52.1285	231.318	−62.21	6.54
8a	172.757	37.1714	209.928	107.04	8.20
8b	172.757	37.1714	209.928	−107.04	8.20
9a	168.113	2.412	170.525	78.87	5.54
9b	168.113	2.412	170.525	−78.87	5.54
10a	160.188	−93.6545	66.534	100.55	6.06
10b	160.188	−93.6545	66.534	−100.55	6.06
11a	104.247	−4.2271	100.020	47.46	6.97
11b	104.247	−4.2271	100.020	−47.46	6.97
12a	135.912	−20.8719	115.04	77.25	–
12b	135.912	−20.8719	115.04	−77.25	–

Vdw. is Van der Waals energy in the minimum energy conformation. Elect. is electrostatic energy in the minimum energy conformation. Dihedral is the dihedral angle ϕ between naphthalene planes in the minimum energy conformation. Distance is the centroid distance of two pharmacophoric side chains in the minimum energy conformation.

Table 4
The key interactions between substrate inhibitors and Bcl-2 protein.

Interactions with Bcl-2	**1a**	**1b**	**2a**	**2b**	**4a**	**4b**	**5b**	**8b**	**10b**	**12b**	(−)-Gossypol
Van der Waals interaction with P1	√	√	√	√	√	√		√	√	√	√
Van der Waals interaction with P2									√		√
Hydrophobic contacts	√	√		√	√	√			√		√
Hydrogen bond (with Y108)							√	√			√
Hydrogen bond (with D111)		√	√	√			√	√			
Hydrogen bond (with F112)		√									
Hydrogen bond (with A113)					√						
Hydrogen bond (with E114)		√			√	√					
Hydrogen bond (with M115)			√								
Hydrogen bond (with S117)			√								
Hydrogen bond (with Q118)	√					√					
Hydrogen bond (with L119)						√					
Hydrogen bond (with V133)								√			
Hydrogen bond (with V134)				√							
Hydrogen bond (with E136)		√	√	√						√	
Hydrogen bond (with D140)										√	
Hydrogen bond (with F153)	√										
Hydrogen bond (with V156)	√										
Hydrogen bond (with E160)					√						√

three cell lines (Table 2). According to their IC_{50} data, we found that when compared with their parent compounds, **12a** and **12b** were more cytotoxic against the HeLa and U87 cancer cell lines but less effective against the M85 cell line, which implied that the elimination of the aldehyde groups did not significantly reduce their anticancer activity. However, after the phenolic groups were protected, the hexamethoxy ethers of gossypol atropisomers **11a** and **11b** were completely inactive. Our experiment confirmed that the phenolic groups play a very important role in the anticancer activity of gossypol atropisomers. Wang and coworkers reported that (−)-gossypol could form a hydrogen bonding network with residues of key proteins such as Bcl-2 through the adjacent hydroxyl groups, and the polyphenolic ring containing three hydroxyl groups could mimic the hydrogen bonding network

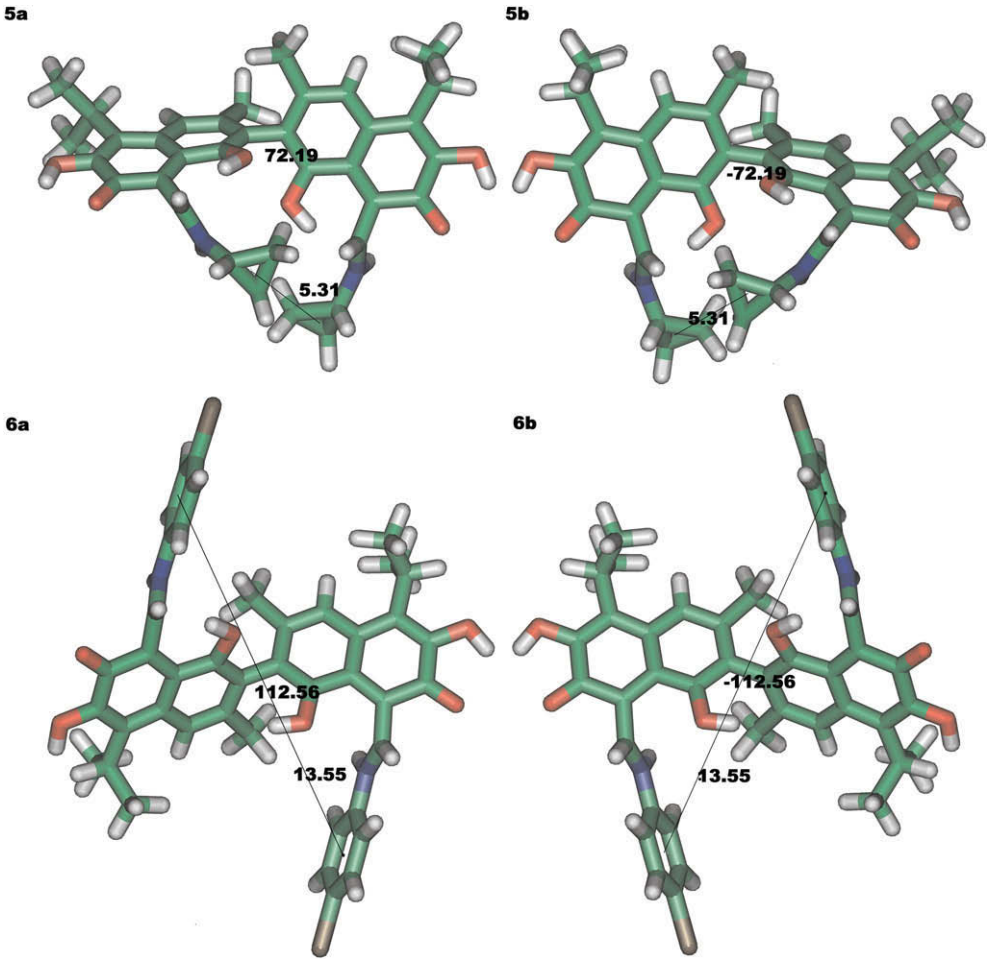


Fig. 2. The minimum energy conformations of compounds **5a**, **5b**, **6a** and **6b** are shown in stick form. The compound number is at the top left corner of each panel.

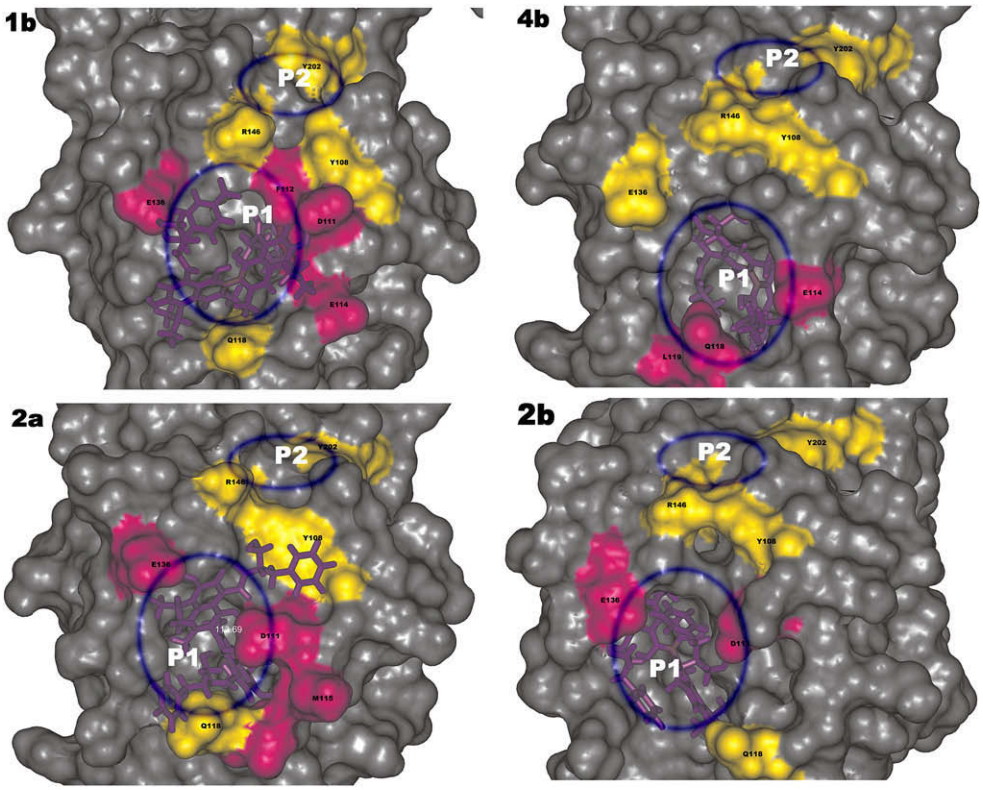


Fig. 3. Binding models of Bcl-2 in complex with compounds **1b**, **4b**, **2a** and **2b**. Bcl-2 protein is shown in gray surface representation. Key residues in the Bcl-2 active site and the residues interacted with substrate inhibitors through hydrogen bond are colored in yellow and pink, respectively. Compounds **1b**, **4b**, **2a** and **2b** are depicted in stick with all atoms colored purple. The compound number is at the top left corner of each panel. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

between (−)-gossypol and Bcl-2 [22]. Perhaps this is the reason that the anticancer activity of gossypol atropisomers was eliminated once the phenolic groups were protected.

In order to evaluate the effect of the oxidation state of gossypol on its cytotoxicity, gossypolone (**13**) (Scheme 2) was synthesized and tested. The rapid racemization of gossypolone made our attempts to produce chiral gossypolone by oxidizing the individual gossypol enantiomers impossible. The optical instability of gossypolone Schiff bases prompted us to abandon preparing them. Therefore, in our assays, only racemic gossypolone was tested, and the difference in cytotoxicity between their (+) and (−) isomers was not determined. When compared with (−)-gossypol, gossypolone was more active against U87 cells (IC_{50}, 26.1 μM), but less active against M85 (IC_{50}, 23.3 μM) and HeLa cells (IC_{50}, 28.3 μM). One should note that the cytotoxicity of gossypolone was less selective when compared with gossypol, and the difference in cytotoxicity between them was very small. The fact that some gossypolone Schiff bases which showed remarkable cytotoxicity could be attributed to the effect of the substituting groups and corresponding molecular volume. We also observed that gossypolone, although preserved frozen in the dark, was degraded in a few days. The instability of gossypolone and its Schiff bases was

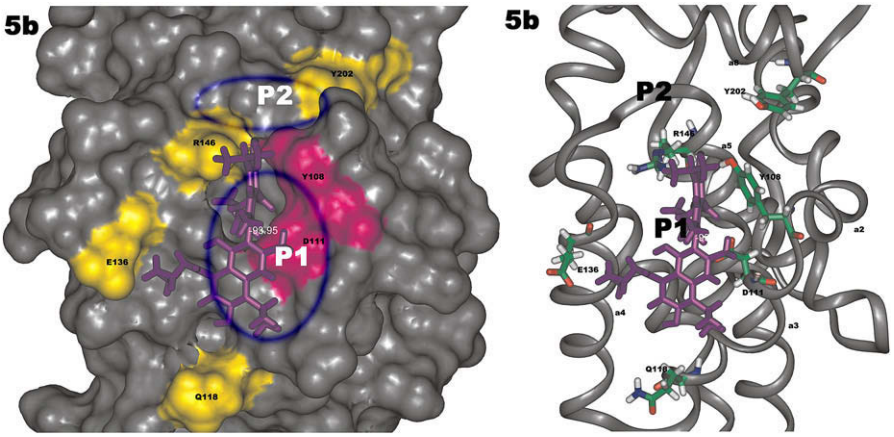


Fig. 4. Schematic representation of Bcl-2 in complex with compound **5b**: Bcl-2 protein is shown in gray surface representation (left); the backbones of Bcl-2 are rendered as gray ribbons (right) with key residues in the Bcl-2 active site and the residues interacted with substrate inhibitors through hydrogen bond showed in stick. Compound **5b** is depicted in stick with all atoms colored purple. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

a problem that limited advanced investigation. Further studies are underway to investigate and ameliorate their instability.

4. Molecular modeling

Bcl-2 family anti-apoptotic members are promising molecular targets for the design of anticancer drugs aimed at overcoming the resistance of cancer cells to apoptosis [33,34]. Non-peptide small molecule inhibitors of Bcl-2 have become a research focus for the development of new cancer agents [35–38]. Recent NMR-derived structure and molecular modeling studies have shown that these proteins consist of a bundle of eight to nine α-helices, which form a structural backbone that is surrounded by six to seven amphipathic α-helices and an elongated hydrophobic groove that forms along the protein surface and spans approximately 20 Å. This serves as the binding site for the amphipathic α-helical BH3 domain of the proapoptotic partners [39,40]. This active site, formed largely by α3, α4 and α5, is mainly divided into two large and deep pockets, P1 and P2, with P1 being the larger one [41].

Recently, studies have shown that (−)-gossypol and its analogs were potent inhibitors of Bcl-2 and Bcl-xL [22–24]. To get a better comprehension of the SAR described above at the molecular level, and to propose binding modes to explain the inhibitory potency, molecular modeling studies and docking experiments were performed. The centroid distance of the two pharmacophoric side chains, the dihedral angle φ between the two naphthalene planes and the electrostatic energy of each compound in the minimum energy conformation were measured, and the results are presented in Table 3. The key interactions between the inhibitors and the side chain of the residues in the Bcl-2 protein in our binding models are depicted in Table 4.

Comparing these data, we observed that compounds with compact conformations, like **1a**, **1b**, **4a** and **4b**, have short distances, high absolute electrostatic energies and strong activity. Compounds **5a** and **5b**, with hydrophobic side chains, have activity that is ascribed to their compact conformations. Conversely, compounds **6a** and **6b**, with extended conformations, have longer distances and lower absolute electrostatic energies as well as corresponding weaker activity on the three cancer cells in comparison with **5a** and **5b** (Fig. 2). Compounds **2a** and **2b**, with extended conformations but strong activity, indicate that the hydrophilic property of the side chain is more important than the conformation for antineoplastic activity.

As expected, the analysis of binding models also gave our hypothesis support. The binding models showed that compounds with hydrophilic side chains replacing the aldehyde groups could increase the hydrogen bonding network and have higher binding affinities to Bcl-2 in comparison with their parent compounds. The amine side chains regulated the binding affinity to Bcl-2 in two ways: first, they affected the conformation of the gossypol framework, and second, their own hydrophilic and bulk properties had significant effects on the binding model by forming hydrogen bonds with residues in Bcl-2 or by interfering with hydrophobic contacts. Compounds with compact conformations, like **1b**, **4b** and **8b**, whose isopropyl groups and aromatic backbone could insert into a hydrophobic pocket in Bcl-2 and form clusters in hydrophobic area P1 because of the large volume of this area, had high binding affinities to Bcl-2 through hydrogen bonding network, hydrophobic contacts and strong Van der Waals interactions. Conversely, compound **2a**, with an extended conformation due to its steric hindrance, lacked such hydrophobic contacts and only bound into the shallow surface of Bcl-2 with a relatively lower binding affinity. Compound **2b**, with an extended conformation that was changed into a compact conformation during the docking experiment, would need extra energy to accomplish this process in order to better fit into the active site (Fig. 3). Compound **10b**, due to its small volume, could interact adequately with the receptor through hydrophobic contacts and strong Van der Waals interactions. Consistent with their antineoplastic activities, these compounds' affinities and binding models showed that maybe they were potential inhibitors of the anti-apoptotic Bcl-2 protein and induced apoptosis through overcoming the resistance of cancer cells to it. Compounds **5a** and **5b**, having selective antineoplastic activity, are hypothesized to induce apoptosis through other ways since **5b** only bound into the shallow surface of Bcl-2 with a relatively low binding affinity, and **5a** could not bind at all (Fig. 4).

5. Conclusion

Our study showed that the low-active form of (+)-gossypol could be changed into a more potent form through a simple chemical modification. Compounds **1b**, **2b**, **4a**, **4b** and **8b** are the most promising candidates because of their exceedingly powerful anticancer activity, and compounds **1a**, **2a**, **5a** and **5b**, even if their IC_{50} values are somewhat higher than (−)-gossypol, should be considered for the following reasons. First, **1a**, **2a** and **5a** are derivatives of (+)-gossypol but had comparable activity with (−)-gossypol. Second, **5a** and **5b** showed strong and selective anticancer activity. Our data confirmed that the anticancer activity of chiral gossypol derivatives depended on the availability of the phenolic groups and on the physicochemical properties of the substituents in the Schiff bases, such as bulk and hydrophilicity. The hydrophilic properties and the bulk of the substituting groups play decisive roles in these Schiff bases' anticancer activity.

It was worth mentioning that compounds **1b**, **4b** and **8b** were remarkably more effective against all three cancer cell lines as compared with the anticancer drug Cisplatin, whereas the cytotoxicity of compounds **2b** and **5b** was more selective, but they had similar effects as Cisplatin on the HeLa and M85 cancer cell lines, and less cytotoxicity against the U87 cell line. These findings provide very valuable clues for the further chemical modification and utilization of gossypol, especially (+)-gossypol. Some of these compounds have the possibility to become very promising therapeutic agents against cancer.

6. Experiment section

6.1. Chemistry

Reagents and solvents used for the reactions were available in the market and were used without further purification. IR spectra using KBr discs were measured on a JASCO FI/IR-480 Plus Fourier Transform spectrometer. Optical rotation was performed on JASCO Polarimeter 1020. Diasteromeric excess or ee values were determined on a SHIMADZU LC-10AT HPLC using a CHIRALPAK AD-H column with flow speed of 1.0 mL/min and detecting wavelength of 254 nm. Melting points were determined on an X-5 hot stage microscope (uncorrected). NMR spectra were recorded on a Varian INOVA NMR 300 or Varian INOVA 500 spectrometer with a constant temperature of 298 K.

6.1.1. (+)-Gossypol and (−)-gossypol

A solution of 200 mg of (+)-gossypol bis-(L-tryptophan methyl ester) Schiff's base in 20 mL of ether and 2 mL of glacial acetic acid was stirred at 50 °C under nitrogen. 60 μL of concentrated hydrochloric acid (37%) was added and the mixture solution was stirred for 1 h. After the white precipitate was filtered off, the filtrate was washed with distilled water until the pH was near 7. Consequently, the solvent was removed at reduced pressure and the residue was dried to give (+)-gossypol as orange crystalline solid in 90% yield

(102 mg). $[\alpha]_D^{25} + 386.44$ (*c* 0.05, $CHCl_3$) (lit. $[\alpha]^{19} + 348.25$ (*c* 0.05, CH_3OH) [18]); HPLC: $R_t = 26.790$ min (ee > 99%, *i*-PrOH/hexane = 30:70 and 0.1% TFA); mp 147–149 °C; ESI-MS: *m*/*z* 517.1 $[M - H]^-$; ^1H NMR (300 MHz, $CDCl_3$): δ 1.54 (d, 12H, $J = 6.9$ Hz, $2 \times (CH_3)_2CH$), 2.15 (s, 6H, $2 \times$ Ar–CH_3), 3.89 (broad m, 2H, $J = 6.9$ Hz, $2 \times (CH_3)_2CH$), 5.99 (s, 2H, $2 \times$ 1-O*H*), 6.39 (s, 2H, $2 \times$ 6-O*H*), 7.78 (s, 2H, $2 \times$ 4-*H*), 11.11 (s, 2H, $2 \times$ C*H*O), 14.42 (s, 2H, $2 \times$ 7-O*H*). (−)-Gossypol, orange crystalline solid, was prepared in 95% yield (108 mg) starting from 200 mg of (−)-gossypol bis-(L-tryptophan methyl ester) Schiff's base by following the same procedure described above. $[\alpha]_D^{25} - 387.6$ (*c* 0.05, $CHCl_3$) (lit. $[\alpha]^{29} - 363.6$ (*c* 0.2, CH_3OH) [18]); HPLC: $R_t = 6.173$ min (ee > 99%, *i*-PrOH/hexane = 30:70 and 0.1% TFA); mp 145–149 °C; ESI-MS: *m*/*z* 517.1 $[M - H]^-$; ^1H NMR (300 MHz, $CDCl_3$): δ 1.54 (d, 12H, $J = 7.5$ Hz, $2 \times (CH_3)_2CH$), 2.14 (s, 6H, $2 \times$ Ar–CH_3), 3.90 (broad m, 2H, $J = 7.5$ Hz, $2 \times (CH_3)_2CH$), 6.07 (s, 2H, $2 \times$ 1-O*H*), 6.36 (s, 6H, $2 \times$ 6-O*H*), 7.77 (s, 2H, $2 \times$ 4-*H*), 11.10 (s, 2H, $2 \times$ C*H*O), 14.98 (s, 2H, $2 \times$ 7-O*H*).

6.1.2. (+)-Gossypol bis ((R,R)-2-amino-1-(4-nitro-phenyl) propane-1,3-diol) Schiff's base (**1a**) and (−)-gossypol bis ((R,R)-2-amino-1-(4-nitro-phenyl) propane-1,3-diol) Schiff's base (**1b**)

A solution of 52 mg of (+)-gossypol in 4 mL of methanol was stirred under nitrogen at room temperature and 53 mg of (*R*,*R*)-2-amino-1-(4-nitro-phenyl) propane-1,3-diol was added. The mixture was stirred for 2 h and dissolved in 1 mL of ethyl acetate after the solvent was removed at reduced pressure. And then, the mixture solution was dropped into 10 mL of cool petroleum ether. The deposit was filtered and washed with 1 mL of cool distilled water and dried. **1a** was given as dark-brown crystalline solid in 95% (86 mg). $[\alpha]_D^{25} + 127.07$ (*c* 0.02, $CHCl_3$); HPLC: $R_t = 31.665$ min (de > 99%, *i*-PrOH/hexane = 12:88); mp 218–222 °C; υ_{max} (KBr)/cm^{-1} 3400–3500 (OH, NH), 1740 (C=O), 1600–1700 (C=C); HR-MS (ESI): *m*/*z* calcd. for $C_{48}H_{50}N_4O_{14}$: 907.3396 $[M + H]^+$; found: 907.3399; ^1H NMR (500 MHz, DMSO-d_6) δ 1.41 ($2 \times$ d, 12H, $J = 7.0$ Hz, $2 \times (CH_3)_2CH$), 1.86 (s, 6H, $2 \times$ Ar–CH_3), 3.55 (broad m, 2H, $J = 7.0$ Hz, $2 \times (CH_3)_2CH$), 3.58 (broad m, 2H, $J = 8.0$ Hz, $2 \times$ 8′-*H*), 3.66 (m, 4H, $2 \times$ 9′-*H*), 3.77 (d, 2H, $2 \times$ 7′-*H*), 5.06 (s, 2H, $2 \times$ 9-O*H*), 5.16 (s, 2H, $2 \times$ 7-O*H*), 6.24 (s, 2H, $2 \times$ 1-O*H*), 7.37 (s, 2H, $2 \times$ 4-*H*), 7.66 (d, 4H, $J = 8.5$ Hz, $2 \times$ 2′,6′-*H*), 8.15 (d, 4H, $J = 8.5$ Hz, $2 \times$ 3′,5′-*H*), 8.47 (s, 2H, $2 \times$ 6-O*H*), 9.52 (d, 2H, $J = 13.0$ Hz, $2 \times$ =C*H*–NH), 13.47 (dd, 2H, $J = 13.0$, 8.0 Hz, $2 \times$ =CH–N*H*). **1b**, Dark-brown crystalline solid, was prepared in 98% yield (89 mg) starting from 52 mg of (−)-gossypol by following the same procedure described for the preparation of **1a**. $[\alpha]_D^{25} - 756.4$ (*c* 0.02, $CHCl_3$); HPLC: $R_t = 33.66$ min (de > 99%, *i*-PrOH/hexane = 12:88); mp 207–210 °C; υ_{max} (KBr)/cm^{-1} 3400–3500 (OH, NH), 1740 (C=O), 1600–1700 (C=C); HR-MS (ESI): *m*/*z* calcd. for $C_{48}H_{50}N_4O_{14}$: 907.3396 $[M + H]^+$; found: 907.3368; ^1H NMR (500 MHz, DMSO-d_6) δ 1.41 ($2 \times$ d, 12H, $J = 7.0$ Hz, $2 \times (CH_3)_2CH$), 1.77 (s, 6H, $2 \times$ Ar–CH_3), 3.55 (broad m, 2H, $J = 7.0$ Hz, $2 \times (CH_3)_2CH$), 3.58 (broad m, 2H, $J = 8.0$ Hz, $2 \times$ 8′-*H*), 3.65 (t, 4H, $2 \times$ 9′-*H*), 3.76 (broad, 2H, $2 \times$ 7′-*H*), 5.06 (s, 2H, $2 \times$ 9-O*H*), 5.16 (s, 2H, $2 \times$ 7-O*H*), 6.23 (s, 2H, $2 \times$ 1-O*H*), 7.34 (s, 2H, $2 \times$ 4-*H*), 7.64 (d, 4H, $J = 8.5$ Hz, $2 \times$ 2′,6′-*H*), 8.14 (d, 4H, $J = 8.5$ Hz, $2 \times$ 3′,5′-*H*), 8.45 (s, 2H, $2 \times$ 6-O*H*), 9.48 (d, 2H, $J = 13.0$ Hz, $2 \times$ =C*H*–NH), 13.41 (dd, 2H, $J = 13.0$, 8.0 Hz, $2 \times$ =CH–N*H*).

6.1.3. (+)-Gossypol bis (R (+)-2-amino-3-phenyl-1-propanol) Schiff's base (**2a**) and (−)-gossypol bis (R (+)-2-amino-3-phenyl-1-propanol) Schiff's base (**2b**)

A solution of 52 mg of (+)-gossypol in 4 mL of methanol was stirred under nitrogen at room temperature and 34 mg of *R* (+)-2-amino-3-phenyl-1-propanol was added. The mixture was stirred for 3 h and dissolved in 1 mL of ethyl acetate after the solvent was removed at reduced pressure. And then the mixture solution was dropped into 10 mL of cool petroleum ether. The deposit was filtered and washed with 1 mL of cool water and dried. **2a** was given as dark-brown crystalline solid in 94% yield (73 mg). $[\alpha]_D^{25} + 613.5$ (*c* 0.02, $CHCl_3$); HPLC: $R_t = 23.832$ min (de > 99%, *i*-PrOH/hexane = 8:92); mp 176–178 °C; υ_{max} (KBr)/cm^{-1} 3300–3460 (OH, NH), 1756 (C=O), 1600–1700 (C=C); HR-MS (ESI): *m*/*z* calcd. for $C_{48}H_{52}N_2O_8$: 785.3796 $[M + H]^+$; found: 785.3794; ^1H NMR (500 MHz, DMSO-d_6) δ 1.41 ($2 \times$ d, 12H, $J = 7.5$ Hz, $2 \times (CH_3)_2CH$), 1.89 (s, 6H, $2 \times$ Ar–CH_3), 2.92 (m, 4H, $2 \times$ 7′-*H*), 3.02 (broad m, 2H, $2 \times$ 8′-*H*), 3.51 (broad m, 2H, $J = 7.5$ Hz, $2 \times (CH_3)_2CH$), 3.67 (m, 4H, $2 \times$ 9′-*H*), 5.25 (s, 2H, $2 \times$ 1-O*H*), 7.16–7.30 (m, 10H, $2 \times$ Ar–*H*), 7.38 (s, 2H, $2 \times$ 4-*H*), 8.35 (s, 2H, $2 \times$ 6-O*H*), 9.87 (broad, 2H, $2 \times$ =C*H*–NH), 13.40 (broad, 2H, $2 \times$ =CH–N*H*). **2b**, Dark-brown crystalline solid, was prepared in 99% yield (76 mg) starting from 52 mg of (−)-gossypol by following the same procedure described for the preparation of **2a**. $[\alpha]_D^{25} - 236.67$ (*c* 0.02, $CHCl_3$); HPLC: $R_t = 24.66$ min (de > 99%, *i*-PrOH/hexane = 8:92,); mp 151–155 °C; υ_{max} (KBr)/cm^{-1} 3300–3460 (OH, NH), 1756 (C=O), 1600–1700 (C=C); HR-MS (ESI): *m*/*z* calcd. for $C_{48}H_{52}N_2O_8$: 785.3796 $[M + H]^+$; found: 785.3773; ^1H NMR (500 MHz, DMSO-d_6) δ 1.41 ($2 \times$ d, 12H, $J = 7.5$ Hz, $2 \times (CH_3)_2CH$), 1.89 (s, 6H, $2 \times$ Ar–CH_3), 2.89 (m, 4H, $2 \times$ 7′-*H*), 3.03 (broad m, 2H, $2 \times$ 8′-*H*), 3.53 (broad m, 2H, $J = 7.5$ Hz, $2 \times (CH_3)_2CH$), 3.66 (m, 4H, $2 \times$ 9′-*H*), 5.25 (s, 2H, $2 \times$ 1-O*H*), 7.16–7.30 (m, 10H, $2 \times$ Ar–*H*), 7.38 (s, 2H, $2 \times$ 4-*H*), 8.35 (s, 2H, $2 \times$ 6-O*H*), 9.95 (broad, 2H, $2 \times$ =C*H*–NH), 13.39 (broad, 2H, $2 \times$ =CH–N*H*).

6.1.4. (+)-Gossypol bis (2-phenylethylamine) Schiff's base (**3a**) and (−)-gossypol bis (2-phenylethylamine) Schiff's base (**3b**)

A suspended solution of 52 mg of (+)-gossypol in 8 mL of toluene was stirred under nitrogen at room temperature and 25 mg of 2-phenylethylamine (27 μL) in a micro-injector was added drop wise. The mixture was stirred for 2 h and dissolved in 1 mL of ethyl acetate after the solvent was removed in vacuo. And then, the mixture solution was dropped into 10 mL of cool petroleum ether. The deposit was filtered and washed with 1 mL of cool distilled methanol and dried. **3a** was given as orange-brown crystalline solid in 67% yield (48 mg). $[\alpha]_D^{25} + 751.6$ (*c* 0.15, $CHCl_3$); HPLC: $R_t = 37.16$ min (ee > 99%, *i*-PrOH/hexane = 10:90); mp 144–147 °C; υ_{max} (KBr)/cm^{-1} 3300–3400 (OH, NH), 1760 (C=O), 1560–1720 (C=C); HR-MS (ESI): *m*/*z* calcd. for $C_{46}H_{48}N_2O_6$: 725.3585 $[M + H]^+$; found: 725.3566; ^1H NMR (500 MHz, DMSO-d_6) δ 1.42 ($2 \times$ d, 12H, $J = 7.0$ Hz, $2 \times (CH_3)_2CH$), 1.93 (s, 6H, $2 \times$ Ar–CH_3), 2.96 (broad m, 4H, $2 \times$ 7′-*H*), 3.68 (broad m, 2H, $J = 7.0$ Hz, $2 \times (CH_3)_2CH$), 3.77 (broad, 4H, $2 \times$ 8′-*H*), 7.18–7.36 (m, 10H, $2 \times$ Ar-*H*), 7.40 (s, 2H, $2 \times$ 4-*H*), 8.32 (s, 2H, $2 \times$ 6-O*H*), 9.79 (broad, 2H, $2 \times$ =C*H*–NH), 13.20 (broad, 2H, $2 \times$ =CH–N*H*). **3b**, orange-brown crystalline solid, was prepared in 73% yield (53 mg) starting from 52 mg of (−)-gossypol by following the same procedure described for the preparation of **3a**. $[\alpha]_D^{25} - 757.4$ (*c* 0.15, $CHCl_3$); HPLC: $R_t = 36.665$ min (ee > 99%, *i*-PrOH/hexane = 10:90,); mp 149–150 °C; υ_{max} (KBr)/cm^{-1} 3300–3400 (OH, NH), 1760 (C=O), 1560–1720 (C=C); HR-MS (ESI): *m*/*z* calcd. for $C_{46}H_{48}N_2O_6$: 725.3585 $[M + H]^+$; found: 725.3552; ^1H NMR (500 MHz, DMSO-d_6) δ 1.42 ($2 \times$ d, 12H, $J = 7.0$ Hz, $2 \times (CH_3)_2CH$), 1.95 (s, 6H, $2 \times$ Ar–CH_3), 2.96 (broad m, 4H, $2 \times$ 7′-*H*), 3.54 (broad m, 2H, $J = 7.0$ Hz, $2 \times (CH_3)_2CH$), 3.77 (broad, 4H, $2 \times$ 8′-*H*), 7.18–7.36 (m, 10H, $2 \times$ Ar–*H*), 7.40 (s, 2H, $2 \times$ 4-*H*), 8.32 (s, 2H, $2 \times$ 6-O*H*), 9.95 (broad, 2H, $2\times$ =C*H*–NH), 13.18 (broad, 2H, $2\times$ =CH–N*H*).

6.1.5. (+)-Gossypol bis (3-amino-1-propanol) Schiff's base (**4a**) and (−)-gossypol bis (3-amino-1-propanol) Schiff's base (**4b**)

A solution of 52 mg of (+)-gossypol in 6 mL of toluene was stirred under nitrogen at room temperature and 15 mg of 3-amino-1-propanol (16 μL) in a micro-injector was added drop wise. The mixture was stirred for 1 h and dissolved in 1 mL of chloroform after

the solvent was removed in vacuo. And then, the mixture solution was dropped into 10 mL of cool petroleum ether. The deposit was filtered and washed with 1 mL of cool distilled water and dried. **4a** was given as brown crystalline solid in 91% (57 mg) yield. $[\alpha]_D^{25}+532.4$ (*c* 0.02, $CHCl_3$); HPLC: $R_t=51.33$ min (ee > 99%, *i*-PrOH/hexane = 10:90); mp 125–129 °C; υ_{max} (KBr)/cm^{-1} 3200–3450 (OH, NH), 1750 (C=O), 1600–1700 (C=C); HR-MS (MALDI/DHB): *m/z* calcd. for $C_{36}H_{44}N_2O_8$: 655.2990 $[M+Na]^+$; found: 655.2984; ^1H NMR (500 MHz, DMSO-d_6) δ 1.43 (2 × d, 12H, $J=7.0$ Hz, 2 × (CH_3)$_2$CH), 1.77 (broad, 4H, 2 × 2′-CH_2), 1.93 (s, 6H, 2 × Ar–CH_3), 3.49 (broad m, 4H, 2 × 1′-CH_2), 3.57 (m, 2H, $J=7.0$ Hz, 2 × (CH_3)$_2$C*H*), 3.69 (m, 4H, 2 × 3′-CH_2), 4.65 (s, 2H, 2 × 1-O*H*), 7.41 (s, 2H, 2 × 4-*H*), 8.36 (s, 2H, 2 × 6-O*H*), 9.78 (d, 2H, $J=11.5$ Hz, 2 × = C*H*–NH), 13.20 (broad d, 2H, $J=11.5$, 2× =CH–N*H*). **4b**, Brown crystalline solid, was prepared in 98% yield (62 mg) starting from 52 mg of (−)-gossypol by following the same procedure described for the preparation of **4a**. $[\alpha]_D^{25}-536.46$ (*c* 0.02, $CHCl_3$); HPLC: $R_t=48.498$ min (ee > 99%, *i*-PrOH/hexane = 10:90); mp 131–133 °C; υ_{max} (KBr)/cm^{-1} 3200–3450 (OH, NH), 1750 (C=O), 1600–1700 (C=C); HR-MS (MALDI/DHB): *m/z* calcd. for $C_{36}H_{44}N_2O_8$: 655.2990 $[M+Na]^+$; found: 655.2989; ^1H NMR (500 MHz, DMSO-d_6) δ 1.42 (2 × d, 12H, $J=7.0$ Hz, 2 × (CH_3)$_2$CH), 1.76 (broad, 4H, 2 × 2′-CH_2), 1.92 (s, 6H, 2 × Ar–CH_3), 3.49 (broad m, 4H, 2 × 1′-CH_2), 3.57 (m, 2H, $J=7.0$ Hz, 2 × (CH_3)$_2$C*H*), 3.69 (m, 4H, 2 × 3′-CH_2), 4.65 (s, 2H, 2 × 1-O*H*), 7.41 (s, 2H, 2 × 4-*H*), 8.38 (s, 2H, 2 × 6-O*H*), 9.77 (d, 2H, $J=11.5$ Hz, 2 × C*H*–NH), 13.20 (broad d, 2H, $J=11.5$, 2 × CH–N*H*).

6.1.6. (+)-Gossypol bis (cyclopropylamine) Schiff's base (**5a**) and (−)-gossypol bis (cyclopropylamine) Schiff's base (**5b**)

A solution of 52 mg of (+)-gossypol in 10 mL of toluene was stirred under nitrogen at room temperature and 14 mg of cyclopropylamine (17 μL) in a micro-injector was added drop wise. The mixture was stirred for 0.5 h and dissolved in 1 mL of chloroform after the solvent was removed in vacuo. And then, the mixture solution was dropped into 10 mL of cool petroleum ether. The deposit was filtered and washed with 1 mL of cool methanol and dried. **5a** was given as light-brown solid in 97% yield (58 mg). $[\alpha]_D^{25}+801.52$ (*c* 0.15, CHCl3); HPLC: $R_t=17.407$ min (ee > 99%, *i*-PrOH/hexane = 15:85 and 0.1% TFA); mp 121–122 °C; υ_{max} (KBr)/cm^{-1} 3300–3400 (OH, NH), 1746 (C=O), 1600–1700 (C=C); HR-MS(MALDI/DHB): *m/z* calcd. for $C_{36}H_{40}N_2O_6$: 619.2779 $[M+Na]^+$; found: 619.2782; ^1H NMR (500 MHz, DMSO-d_6) δ 0.83, 0.90 (broad m, 8H, 2 × 2′, 3′-CH_2), 1.43 (2 × d, 12H, $J=7.0$ Hz, 2 × (CH_3)$_2$CH), 1.93 (s, 6H, 2 × Ar–CH_3), 3.20 (broad, 2H, 2 × 1′-C*H*NHCH =), 3.71 (broad m, 2H, $J=7.0$ Hz, 2 × (CH_3)$_2$C*H*), 7.45 (s, 2H, 2 × 4-*H*), 7.91 (s, 2H, 2 × 1-O*H*), 8.37 (s, 2H, 2 × 6-O*H*), 9.87 (d, 2H, $J=10.0$ Hz, 2× =C*H*–NH), 13.54 (broad d, 2H, $J=10.0$, 2 × = CH–N*H*). **5b**, Light-brown powder, was prepared in 99% yield (59 mg) starting from 52 mg of (−)-gossypol by following the same procedure described for the preparation of **5a**. $[\alpha]_D^{25}-807.33$ (*c* 0.15, $CHCl_3$); HPLC: $R_t=9.157$ min (ee > 99%, *i*-PrOH/hexane = 15:85 and 0.1% TFA); mp 113–117 °C; υ_{max} (KBr)/cm^{-1} 3300–3400 (OH, NH), 1746 (C=O), 1600–1700 (C=C); HR-MS (MALDI/DHB): *m/z* calcd. for $C_{36}H_{40}N_2O_6$: 597.2959 $[M+H]^+$; found: 597.2951; ^1H NMR (500 MHz, DMSO-d_6) δ 0.81, 0.89 (broad m, 8H, 2 × 2′, 3′-CH_2), 1.43 (2 × d, 12H, $J=7.0$ Hz, 2 × (CH_3)$_2$CH), 1.93 (s, 6H, 2 × Ar–CH_3), 3.20 (broad, 2H, 2 × 1′-C*H*NHCH=), 3.70 (broad m, 2H, $J=7.0$ Hz, 2 × (CH_3)$_2$C*H*), 7.45 (s, 2H, 2 × 4-*H*), 7.91 (s, 2H, 2 × 1-O*H*), 8.36 (s, 2H, 2 × 6-O*H*), 9.86 (d, 2H, $J=10.0$ Hz, 2× =C*H*–NH), 13.53 (broad d, 2H, $J=10.0$, 2× =CH–N*H*).

6.1.7. (+)-Gossypol bis (4-bromo-phenylamine) Schiff's base (**6a**) and (−)-gossypol bis (4-bromo-phenylamine) Schiff's base (**6b**)

52 mg of (+)-gossypol was dissolved in a solution of 6 mL of chloroform and 70 mg of 4-bromo-phenylamine was added. The mixture was stirred under nitrogen for 12 h at room temperature. **6a** was given as orange powder in 28% yield (23 mg) from the separation of the mixture by column chromatograph ($R_f=0.65$ in 3:1 petroleum ether/chloroform). $[\alpha]_D^{25}+741.57$ (*c* 0.02, $CHCl_3$); HPLC: $R_t=12.50$ min (ee > 99%, *i*-PrOH/hexane = 8:92); mp 142–149 °C; υ_{max} (KBr)/cm^{-1} 3400–3600 (OH, NH), 1768 (C=O), 1600–1700 (C=C); HR-MS (MALDI/DHB): *m/z* calcd. for $C_{36}H_{40}N_2O_6$: 825.1169 $[M+H]^+$; found: 825.1163; ^1H NMR (500 MHz, $CDCl_3$) δ 1.55 (2 × d, 12H, $J=7.0$ Hz, 2 × (CH_3)$_2$CH), 2.15 (s, 6H, 2 × Ar–CH_3), 3.74 (broad m, 2H, $J=7.0$ Hz, 2 × (CH_3)$_2$C*H*), 5.75 (s, 2H, 1-O*H*), 7.29 (d, 4H, $J=7.5$ Hz, 2 × 2′,6′-*H*), 7.36 (d, 4H, $J=7.5$ Hz, 2 × 3′,5′-*H*), 7.63 (s, 2H, 2 × 4-*H*), 7.89 (s, 2H, 2 × 6-O*H*), 10.17 (d, 2H, $J=10.0$ Hz, 2× =C*H*–NH), 14.91 (broad d, 2H, $J=10.0$, 2× =CH–N*H*). **6b**, Orange powder, was prepared in 57% yield (47 mg) starting from 52 mg of (−)-gossypol by following the same procedure described for the preparation of **6a**. $[\alpha]_D^{25}-740.35$ (*c* 0.02, $CHCl_3$); HPLC: $R_t=10.165$ min (ee > 99%, *i*-PrOH/hexane = 8:92); mp 142–145 °C; υ_{max} (KBr)/cm^{-1} 3400–3600 (OH, NH), 1768 (C=O), 1600–1700 (C=C); HR-MS (MALDI/DHB): *m/z* calcd. for $C_{36}H_{40}N_2O_6$: 825.1169 $[M+H]^+$; found: 825.1165; ^1H NMR (500 MHz, $CDCl_3$) δ 1.55 (2 × d, 12H, $J=7.0$ Hz, 2 × (CH_3)$_2$CH), 2.16 (s, 6H, 2 × Ar–CH_3), 3.74 (broad m, 2H, $J=7.0$ Hz, 2 × (CH_3)$_2$C*H*), 5.79 (s, 2H, 1-O*H*), 7.30 (d, 4H, $J=7.5$ Hz, 2 × 2′,6′-*H*), 7.37 (d, 4H, $J=7.5$ Hz, 2 × 3′,5′-*H*), 7.63 (s, 2H, 2 × 4-*H*), 7.89 (s, 2H, 2 × 6-O*H*), 10.18 (d, 2H, $J=11.0$ Hz, 2× =C*H*–NH), 14.91 (broad d, 2H, $J=11.0$, 2× =CH–N*H*).

6.1.8. (+)-Gossypol bis-(L-phenylalanine methyl ester) Schiff's base (**7a**) and (−)-gossypol bis-(L-phenylalanine methyl ester) Schiff's base (**7b**)

A solution of 43 mg of L-phenylalanine methyl ester hydrochloride and 8 mg of sodium hydroxide in 6 mL of methanol was stirred at room temperature under nitrogen and the pH was near 9. And then 52 mg of (+)-gossypol was added and the solution was stirred at 55 °C for 4 h. Consequently, the solvent was removed at reduced pressure and the residue was washed with chloroform and filtered off. The filtrate was concentrated and dropped into 10 mL of cool petroleum ether. The deposit was filtered and washed with 1 mL of cool distilled water and dried. **7a** was given as yellow crystalline solid in 93% yield (78 mg). $[\alpha]_D^{25}+569.38$ (*c* 0.02, acetone) (lit. $[\alpha]_D^{25}+514.9$ (*c* 0.09, DCM) [21]); mp 120–123 °C; ESI-MS: *m/z* 841.5 $[M+H]^+$; ^1H NMR (300 MHz, $CDCl_3$) δ 1.53 (2 × d, 12H, $J=6.6$ Hz, 2 × (CH_3)$_2$CH), 2.05 (s, 6H, 2 × Ar–CH_3), 3.13 (dd, 2H, $J=14.1$, 9.0 Hz, 2 × CH_2-a), 3.33 (dd, 2H, $J=14.1$, 5.1 Hz, 2 × CH_2-b), 3.71 (broad m, 2H, $J=6.6$ Hz, 2 × (CH_3)$_2$C*H*), 3.77 (s, 6H, 2 × $COOCH_3$), 4.28 (dd, 2H, $J=9.0$, 5.1 Hz, 2 × α-C*H*), 5.31 (s, 2H, 2 × 1-O*H*), 7.16–7.25 (m, 10H, 2 × Ar–*H*), 7.55 (s, 2H, 2 × 4-*H*), 7.94 (s, 2H, 2 × 6-O*H*), 9.24 (d, 2H, $J=11.7$ Hz, 2× =C*H*–NH), 13.60 (broad d, 2H, $J=11.7$ Hz, 2× =CH–N*H*). **7b**, yellow crystalline solid was prepared in 97% yield (81 mg) starting from 52 mg of (−)-gossypol by following the same procedure described for the preparation of **7a**. $[\alpha]_D^{25}-673.79$ (*c* 0.02, acetone) (lit. $[\alpha]_D^{25}-838.9$ (*c* 0.12, DCM) [21]); mp 118–121 °C; ESI-MS: *m/z* 841.5 $[M+H]^+$; ^1H NMR (300 MHz, $CDCl_3$) δ 1.53 (2 × d, 12H, $J=6.9$ Hz, 2 × (CH_3)$_2$CH), 2.06 (s, 6H, 2 × Ar–CH_3), 3.17 (dd, 2H, $J=13.5$, 8.7 Hz, 2 × CH_2-a), 3.34 (dd, 2H, $J=13.5$, 4.8 Hz, 2 × CH_2-b), 3.71 (broad m, 2H, $J=6.9$ Hz, 2 × (CH_3)$_2$C*H*), 3.78 (s, 6H, 2 × $COOCH_3$), 4.32 (dd, 2H, $J=8.7$, 4.8 Hz, 2 × α-C*H*), 7.13–7.25 (m, 10H, 2 × Ar–*H*), 7.54 (s, 2H, 2 × 4-*H*), 7.94 (s, 2H, 2 × 6-O*H*), 9.38 (d, 2H, $J=12.0$ Hz, 2× =C*H*–NH), 13.59 (broad d, 2H, $J=12.0$ Hz, 2× =CH–N*H*).

6.1.9. (+)-Gossypol bis-(L-tyrosine methyl ester) Schiff's base (**8a**) and (−)-gossypol bis-(L-tyrosine methyl ester) Schiff's base (**8b**)

8a, Light-brown crystalline solid was prepared in 97% yield (84 mg) starting from 52 mg of (−)-gossypol and L-tyrosine methyl ester hydrochloride by following the same procedure described for

the preparation of **7a**. $[\alpha]_D^{25}$ + 354.31 (*c* 0.02, CH_3OH), mp 127–131 °C; ESI-MS: *m/z* 895.7 $[M + Na]^+$; ^1H NMR (500 MHz, DMSO-d_6) δ 1.42 (2 × d, 12H, *J* = 7.0 Hz, 2 × $(CH_3)_2CH$), 1.91 (s, 6H, 2 × Ar–CH_3), 3.09 (dd, 2H, *J* = 14.5 Hz, 2 × CH_2-a), 3.11 (dd, 2H, *J* = 14.5 Hz, 2 × CH_2-b), 3.66 (broad m, 2H, *J* = 7.0 Hz, 2 × $(CH_3)_2CH$), 3.67 (s, 6H, 2 × $COOCH_3$), 4.71 (m, 2H, 2 × α-CH), 6.62 (dd, 4H, *J* = 8.0, 2.0 Hz, 2 × 3′,5′-H), 6.97 (dd, 4H, *J* = 8.0, 2.0 Hz, 2 × 2′,6′-H), 7.41 (s, 2H, 2 × 4-H), 7.89, 8.44 (s, 4H, 4 × -OH), 9.28 (s, 2H, 2 × 4′-OH), 9.73 (d, 2H, *J* = 12.0 Hz, 2× =CH–NH), 13.41 (broad m, 2H, *J* = 12.0 Hz, 2× =CH–NH). **8b**, Light-brown crystalline solid was prepared in 98% yield (86 mg) starting from 52 of mg (−)-gossypol by following the same procedure described for the preparation of **7a**. $[\alpha]_D^{25}$ − 753.98 (*c* 0.02, CH_3OH), mp 123–125 °C; ESI-MS: *m/z* 895.7 $[M + Na]^+$; ^1H NMR (500 MHz, DMSO-d_6) δ 1.42 (d, 12H, *J* = 7.5 Hz, 2 × $(CH_3)_2CH$), 1.92 (s, 6H, 2 × Ar–CH_3), 3.07 (dd, 2H, *J* = 14.5, 5.5 Hz, 2 × CH_2-a), 3.13 (dd, 2H, *J* = 14.5, 5.5 Hz, 2 × CH_2-b), 3.67 (broad m, 2H, *J* = 7.5 Hz, 2 × $(CH_3)_2CH$), 3.68 (s, 6H, 2 × $COOCH_3$), 4.68 (m, 2H, 2 × α-CH), 6.63 (dd, 4H, *J* = 8.0, 2.0 Hz, 2 × 3′,5′-H), 6.97 (dd, 4H, *J* = 8.0, 2.0 Hz, 2 × 2′,6′-H), 7.42 (s, 2H, 2 × 4-H), 7.82, 8.44 (s, 4H, 4 × -OH), 9.27 (s, 2H, 2 × 4′-OH), 9.67 (d, 2H, *J* = 12.0 Hz, 2× =CH–NH), 13.42 (broad m, 2H, *J* = 12.0 Hz, 2× =CH–NH).

6.1.10. (+)-Gossypol bis-(L-tryptophan methyl ester) Schiff's base (**9a**) and (−)-gossypol bis-(L-tryptophan methyl ester) Schiff's base (**9b**)

A solution of 500 mg of L-tryptophan methyl ester hydrochloride and 80 mg of sodium hydroxide in 100 mL of methanol was stirred at room temperature under nitrogen and the pH was near 9. And then 500 mg of racemic gossypol was added and the solution was stirred at 55 °C for 4 h. Consequently, the solvent was removed at reduced pressure and the residue was washed with chloroform and filtered off. The filtrate was concentrated to give the diastereoisomeric mixture. The separation of diastereoisomeric mixture by rapid column chromatograph, **9b** was eluted firstly and recrystallized from hexane/ethyl acetate (70:30) as brown crystalline solid (436 mg, 44% yield) and **9a** was eluted secondly and recrystallized from chloroform as brown crystalline solid (357 mg, 36% yield). **9a**, $[\alpha]_D^{25}$ + 273.54 (*c* 0.02, CH_3OH) (lit. $[\alpha]^{18.5}$ + 250.0 (*c* 0.02, CH_3OH) [21]); mp 143–148 °C; ESI-MS: *m/z* 917.3 $[M - H]^-$; ^1H NMR (500 MHz, DMSO-d_6) δ 1.42 (2 × d, 12H, *J* = 7.0 Hz, 2 × $(CH_3)_2CH$), 1.91 (s, 6H, 2 × Ar–CH_3), 3.35 (broad m, 4H, 2 × CH_2), 3.65 (s, 6H, 2 × $COOCH_3$), 3.62 (broad m, 2H, *J* = 7.0 Hz, 2 × $(CH_3)_2CH$), 4.78 (m, 2H, 2 × α-CH), 6.91 (d, 2H, *J* = 8.0 Hz, 2 × 5′-H), 6.98 (t, 2H, *J* = 8.0 Hz, 2 × 6′-H), 7.13 (d, 2H, *J* = 2.0 Hz, 2 × 2′-H), 7.29 (d, 2H, *J* = 8.0 Hz, 2 × 7′-H), 7.43 (s, 2H, 2 × 4-H), 7.46 (d, 2H, *J* = 8.0 Hz, 2 × 4′-H), 7.64, 8.44 (s, 4H, 4 × –OH), 9.64 (d, 2H, *J* = 12.5 Hz, 2× =CH–NH), 10.92 (d, 2H, *J* = 2.0 Hz, 2 × indole NH), 13.45 (dd, 2H, *J* = 12.5, 8.0 Hz, 2× =CH–NH). 9b, $[\alpha]_D^{25}$ −754.37 (*c* 0.02, CH_3OH) (lit. $[\alpha]^{21}$ − 1175.2 (*c* 0.05, DCM) [21]); mp 176–180 °C; ESI-MS: *m/z* 917.3 $[M - H]^-$; ^1H NMR (500 MHz, DMSO-d_6) δ 1.42 (2 × d, 12H, *J* = 7.5 Hz, 2 × $(CH_3)_2CH$), 1.92 (s, 6H, 2 × Ar–CH_3), 3.35 (broad m, 4H, 2 × CH_2), 3.66 (s, 6H, 2 × $COOCH_3$), 3.67 (broad m, 2H, *J* = 7.5 Hz, 2 × $(CH_3)_2CH$), 4.80 (m, 2H, 2 × α-CH), 6.93 (d, 2H, *J* = 8.0 Hz, 2 × 5′-H), 7.03 (t, 2H, *J* = 8.0 Hz, 2 × 6′-H), 7.13 (d, 2H, *J* = 2.0 Hz, 2 × 2′-H), 7.30 (d, 2H, *J* = 8.0 Hz, 2 × 7′-H), 7.44 (s, 2H, 2 × 4-H), 7.46 (d, 2H, *J* = 8.0 Hz, 2 × 4′-H), 7.82, 8.44 (s, 4H, 4 × –OH), 9.76 (d, 2H, *J* = 12.5 Hz, 2× =CH–NH), 10.93 (d, 2H, *J* = 2.0 Hz, 2 × indole NH), 13.47 (dd, 2H, *J* = 12.5, 8.0 Hz, 2× =CH–NH). The NMR data of these two compounds were consistent with the previous report [21].

6.1.11. (+)-Gossypol bis (isopropylamine) Schiff's base (**10a**) and (−)-gossypol bis (isopropylamine) Schiff's base (**10b**)

A solution of 52 mg of (+)-gossypol in 8 mL of ether was stirred under nitrogen at room temperature and 50 μL of cyclopropylamine in a micro-injector was added drop wise. The reaction was completed in 0.5 h and **10a**, after the solvent was removed at reduced pressure, was given as orange powder in 99% yield (60 mg). $[\alpha]_D^{25}$ + 738.23 (*c* 0.02, $CHCl_3$); HPLC: R_t = 16.16 min (ee 95%, *i*-PrOH/hexane = 11:89); mp 112–115 °C; ESI-MS: *m/z* 601.1 $[M + H]^+$; ^1H NMR (300 MHz, $CDCl_3$) δ 1.40 (2 × d, 12H, *J* = 4.8 Hz, 2 × $(CH_3)_2$ CH–NH), 1.53 (2 × d, 12H, *J* = 6.9 Hz, 2 × $(CH_3)_2CH$), 2.12 (s, 6H, 2 × Ar–CH_3), 3.72 (broad m, 2H, *J* = 4.8 Hz, $(CH_3)_2CH$–NH), 3.76 (broad m, 2H, *J* = 6.9 Hz, 2 × $(CH_3)_2CH$), 5.59 (s, 2H, 2 × 1-OH), 7.61 (s, 2H, 2 × 4-H), 8.03 (s, 2H, 2 × 6-OH), 9.71 (d, 2H, *J* = 12.6 Hz, 2 × $(CH_3)_2CH$–NH–CH=), 13.42 (broad d, 2H, *J* = 12.6 Hz, 2 × $(CH_3)_2CH$–NH–CH=). **10b**, Bright orange powder was prepared in 99% yield (60 mg) starting from 52 mg (−)-gossypol by following the same procedure described for the preparation of **10a**. $[\alpha]_D^{25}$ − 736.94 (*c* 0.02, $CHCl_3$); HPLC: R_t = 8.49 min (ee 96%, *i*-PrOH/hexane = 11:89); mp 117–121 °C; ESI-MS: *m/z* 601.1 $[M + H]^+$; ^1H NMR (300 MHz, $CDCl_3$) δ 1.39 (t, 12H, 2 × $(CH_3)_2CH$–NH), 1.54 (2 × d, 12H, *J* = 8.1 Hz, 2 × $(CH_3)_2CH$), 2.12 (s, 6H, 2 × Ar–CH_3), 3.71 (broad m, 2H, $(CH_3)_2CH$-NH), 3.76 (broad m, 2H, *J* = 8.1 Hz, 2 × $(CH_3)_2CH$), 5.61 (s, 2H, 2 × 1-OH), 7.61 (s, 2H, 2 × 4-H), 8.04 (s, 2H, 2 × 6-OH), 9.72 (d, 2H, *J* = 12.3 Hz, 2 × $(CH_3)_2CH$–NH–CH=), 13.39 (broad d, 2H, *J* = 12.3 Hz, 2 × $(CH_3)_2CH$–NH–CH=).

6.1.12. (+)-5,5′-Diisopropyl-2,3,4,2′,3′,4′-hexamethoxy-7,7′-dimethyl-2H,2H′-[8,8′] bi [naphtha [1,8-bc] furanyl] (**11a**, (+)-gossypol hexamethyl ether) and (−)-5,5′-diisopropyl-2,3,4,2′,3′,4′-hexamethoxy-7,7′-dimethyl-2H,2H′-[8,8′] bi [naphtha [1,8-bc] furanyl] (**11b**, (−)-gossypol hexamethyl ether)

A solution of 52 mg of (+)-gossypol in 4 mL of absolute methanol was stirred at room temperature under nitrogen. When solution was completed, a solution of 1 mL of dimethyl sulfate was added drop wise and the mixture was allowed to stir for 0.5 h. Consequently, a solution of 4 M potassium hydroxide was added drop wise with stirring to maintain the pH of the solution near 9. About 4 h later, the reaction was completed and the final pH of the mixture solution was 8–9. Consequently, 5 mL of chloroform was added and the solvent was removed at reduced pressure after the sodium sulfate was filtered off. The white residue was washed with cool methanol and dried to give **11a** in 58% yield (35 mg). $[\alpha]_D^{25}$ + 85.07 (*c* 0.02, $CHCl_3$); mp 133–138 °C; ESI-MS: *m/z* 625.1 $[M + Na]^+$; ^1H NMR (500 MHz, $CDCl_3$) δ 1.54 (2 × d, 12H, *J* = 7.5 Hz, 2 × $(CH_3)_2CH$), 2.31 (s, 6H, 2 × Ar–CH_3), 3.48 (broad, 6H, 2 × O–CH–OCH_3), 3.87 (broad m, 2H, *J* = 7.5 Hz, 2 × $(CH_3)_2CH$), 4.10 (s, 12H, 4 × –OCH_3), 7.05 (broad, 2H, O–CH–OCH_3), 7.47 (s, 2H, 2 × 4-H). **11b**, A white solid was prepared in 63% yield (38 mg) starting from 52 mg of (−)-gossypol by following the same procedure described for the preparation of **11a**. $[\alpha]_D^{25}$ − 90.34 (*c* 0.02, $CHCl_3$); mp 137–139 °C; ESI-MS: *m/z* 625.1 $[M + Na]^+$; ^1H NMR (500 MHz, $CDCl_3$) δ 1.54 (2 × d, 12H, *J* = 7.5 Hz, 2 × $(CH_3)_2CH$), 2.31 (s, 6H, 2 × Ar–CH_3), 3.48 (s, 6H, 2 × O–CH–OCH_3), 3.87 (broad m, 2H, *J* = 7.5 Hz, 2 × $(CH_3)_2CH$), 4.13 (s, 12H, 4-OCH_3), 7.05 (broad, 2H, O–CH–OCH_3), 7.46 (s, 2H, 2 × 4-H).

6.1.13. (+)-5,5′-Diisopropyl-3,3′-dimethyl-2,2′-binaphthyl-1,1′,6,6′,7,7′-hexaol (**12a**, (+)-apogossypol) and (−)-5,5′-diisopropyl-3,3′-dimethyl-2,2′-binaphthyl-1,1′,6,6′,7,7′-hexaol (**12b**, (−)-apogossypol)

A solution of 100 mg of (+)-gossypol acetic acid and 5 mL of 40% aqueous sodium hydroxide was heated for 3 h at 90 °C under nitrogen. Consequently, 3 mL of 10 M cool sulfuric acid was added and the pH of solution was near 1. The resultant precipitate was extracted with ether and recrystallized to give **12a** ((+)-apogossypol, 45 mg). $[\alpha]_D^{25}$ + 133.11 (*c* 0.02, $CHCl_3$); mp 118–123 °C; HR-MS(ESI): *m/z* calcd. for $C_{28}H_{30}O_6$: 485.1935 $[M + Na]^+$; found: 485.1942; ^1H NMR (300 MHz, $CDCl_3$) δ 1.50 (2 × d, 12H, *J* = 6.9 Hz, 2 × $(CH_3)_2CH$), 2.08 (s, 6H, 2 × Ar–CH_3), 3.83 (m, 2H, *J* = 6.9 Hz, 2 × $(CH_3)_2CH$), 5.38, 6.08, 6.57 (broad s, 6H, 2 × 1, 6, 7-OH), 7.36

(s, 2H, 2 × 8-*H*), 7.59 (s, 2H, 2 × 4-*H*). **12b** ((−)-Apogossypol, 53 mg), white solid, was prepared by following the same procedure described for the preparation of **12a**. $[\alpha]_D^{25}$ − 157.46 (*c* 0.02, $CHCl_3$); mp 115–118 °C; ESI-MS: *m*/*z* 461.2 $[M-H]^-$; ^1H NMR (300 MHz, $CDCl_3$) δ 1.50 (2 × d, 12H, $J = 6.6$ Hz, 2 × $(CH_3)_2CH$), 2.08 (s, 6H, 2 × Ar–CH_3), 3.83 (m, 2H, $J = 6.6$ Hz, 2 × $(CH_3)_2CH$), 5.38, 6.08, 6.59 (broad s, 6H, 2 × 1, 6, 7-O*H*), 7.36 (s, 2H, 2 × 8-*H*), 7.58 (s, 2H, 2 × 4-*H*).

6.1.14. 6,6′,7,7′-Tetrahydroxyl-5,5′-diisopropyl-3,3′-dimethyl-1,1′,4,4′-tetraoxo-1,1′,4,4′-tetrahydro-[2,2′]-binaphthyl-8,8′-dicarbaldehyde (**13**, gossypolone)

A solution of 200 mg of (−)-gossypol in 10 mL of acetone and 20 mL acetic acid was stirred at 65 °C during the addition of 10 mL of 10% aqueous solution of ferric chloride. About 40 min later, the solution was cooled and 30 mL of water was added. The precipitate was filtered and treated with a mixture of ether and aqueous 20% sulfuric acid. The ether layer was washed with water and dried (Na_2SO_4) and the residue, after the solvent was removed, was recrystallized in petroleum ether/CH_2Cl_2 (30:70) to give **13** as dark-purple crystalline solid in 42% yield (88 mg). Mp 123–129 °C; ESI-MS: *m*/*z* 545.7 $[M-H]^-$; ^1H NMR (500 MHz, $CDCl_3$) δ 1.45 (2 × d, 12H, $J = 6.6$ Hz, 2 × $(CH_3)_2CH$), 2.06 (s, 6H, 2 × Ar–CH_3), 4.13 (broad m, 2H, $J = 6.6$ Hz, 2 × $(CH_3)_2CH$), 6.60 (s, 2H, 2 × 6-O*H*), 10.60 (s, 2H, 2 × C*H*O), 13.03 (s, 2H, 2 × 7-O*H*).

6.2. Biological evaluation

The assay relied upon the spectrophotometric measurement of solubilized purple formazan, produced by the mitochondrial reduction of MTT in viable cells. First, cancer cell lines (5000 per well) were seeded into 96-well plates and incubated for 24 h (37 °C, 95% air/5% CO_2). Then, medium containing 2.5 μM, 5 μM, 10 μM, 20 μM, 40 μM and 80 μM of the tested compounds (compounds dissolved in DMSO were added to the culture medium, and the control medium received an equivalent dilution of DMSO; the final concentration of DMSO was <1%, with four wells for each experiment concentration) were introduced into wells. After incubation for 48 h, 20 μL of MTT was added into each well, and the cancer cell lines were incubated for 4 h. Consequently, the culture medium containing MTT was removed, and 100 μL of DMSO was added into each well. The absorbance was tested with a multi-scan spectrophotometer MK3 (Thermo Electron Corporation) at 492 nm. Each compound's IC_{50} value was calculated using the Probit analysis program in SPSS10.0. For the purpose of comparison, the activities of (−)-gossypol, (+)-gossypol and anticancer drug Cisplatin were also determined using the same method described above.

6.3. Molecular modeling

The 3D structure of Bcl-2 (1YSW) was obtained from the RCSB Protein Data Bank. All the molecular modeling calculations were performed on an Origin 2000 workstation running the Irix 6.5 operating system (Silicon Graphics Inc., Mountain View, CA, USA) and the Insight II 2000 software package.

The initial random molecular structures were first built in Biopolymer. Their geometry was then optimized with molecular dynamics, and energy minimization was performed on a Discover 98 module under the Insight II 2000 package using the standard protocols. After optimization, further structural analysis on the van der Waals energy, electrostatic energy and the centroid distance of the two pharmacophoric side chains and the dihedral angle ϕ between the two naphthalene planes in the minimum energy conformation were measured.

The flexible ligand docking procedure in the Affinity module within the Insight II 2000 package was used. The 1YSW structure after energy minimization was used as the protein template. All the atoms of the inhibitors were allowed to move freely, and the backbone atoms of the residues, where the substrate bound, were allowed to move with restrictions. The resulting structure was accepted on the basis of energy checks, which used the metropolis criterion. The final conformation was obtained through a simulated annealing procedure at 500 K, followed by 1000 rounds of energy minimization to reach a convergence. The final binding modes of the inhibitors were determined on the basis of the energy.

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