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Original article

Synthesis and cytotoxicity of gossypol related compounds

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Abstract – Gossypol, gossypolone, reduced gossypol and new Schiff's bases of racemic gossypol and gossypolone were extracted or synthesized. Their cytotoxic activities on KB human cancer cells were determined. Gossypolone and the ethylamine derivative of gossypolone were the most active compounds (IC_{50} in the micromolar range in both cases). The cytotoxicity of gossypol and gossypolone was increased when the tests were performed in the absence of serum and decreased when catalase as well as mannitol were added to the culture medium.

gossypol / gossypolone / Schiff's bases / reduced gossypol / oxygen radical scavengers / cytotoxicity

1. Introduction

Gossypol [1,1′,6,6′,7,7′-hexahydroxy-5,5′diisopropyl-3,3′dimethyl- [2,2′] binaphthalenyl-[8,8′] dicarbaldehyde] is a polyphenolic dialdehyde (*figure 1*), a naturally occurring yellow pigment found in high concentrations in the pigment glands of the cotton plant gossypium [1]. Extensive information is available concerning the toxicology and pharmacokinetics of this compound since it has already been administered to humans as a male oral contraceptive [2].

Gossypol and some of its derivatives also display several potentially useful biological activities: antitrypanosomal [3], antimalarial [4–6], antitumour [7–9] and inhibition of enveloped viruses [10–12] including HIV [13–18].

While being marginally toxic, gossypol represents a potential starting point for the development of antiviral or antitumour drugs.

Numerous studies suggest that modifications to gossypol's functional aldehyde groups could reduce its host toxicity while retaining its therapeutic effects [15, 16]. The availability of stable derivatives of gossypol, in which only the aldehyde groups are modified, has been limited because the phenolic hydroxyl groups in the 1 and 1′ (*peri*) positions complicate the chemistry of the functional group at the 8 and 8′ positions [1].

Nevertheless, the reaction of gossypol with primary amines, giving gossypol imines or Schiff's bases has been extensively developed and, in the meantime, the broad biological activity of gossypol has been explained by its easy coupling to lysine side chains of proteins [1] and to enzymes involved in the cellular growth process [19].

Gossypol imines have been investigated for their antitumour or inhibitory effects and some structure–activity comparisons have already been proposed pointing out the importance of the substituting group [8, 20].

More recently, the metabolic fate of gossypol was reinvestigated in view of the toxicity of gossypolone. As a polyphenol, gossypol has been cited as a promoter of free radical formation in biological media and part of its cytotoxicity has been attributed also to its transformation into gossypolone which represents the major metabolite of gossypol in vivo [21, 22].

In the present paper, we describe the synthesis of several original derivatives of gossypol and gossypolone and their cytotoxic activities on the KB human cancer cell line. Furthermore, to get some insight into the role played by oxidation in cytotoxicity, we investigated the activity

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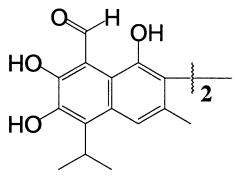
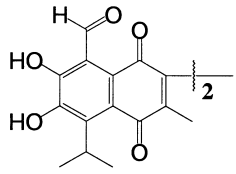


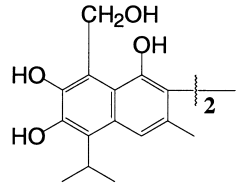
Gossypol 1

(5.7)



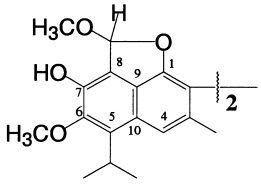
Gossypolone 2

(2.4)



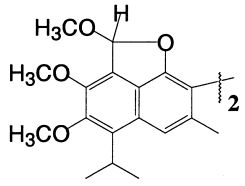
Gossypolol 3

(21.8)



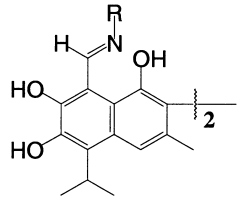
4

(>25)

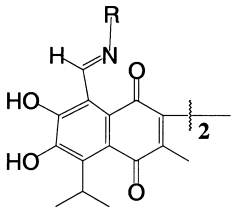


5

(>25)



(10.4)	**6** R = CH_3
(5.6)	**7** = C_2H_5
(6.5)	**8** = C_3H_7
(6.6)	**9** = $CH(CH_3)_2$
(12.4)	**10** = C_4H_9
(6.7)	**11** = CH_2-$CH(CH_3)_2$
(13.5)	**12** = $C(CH_3)_3$
(>20)	**13** = C_5H_{11}
(>20)	**14** = C_6H_{13}
(>20)	**15** = C_7H_{15}
(>20)	**16** = CH_2-$CH(CH_3)C_6H_5$
(>20)	**17** = $CH(COOCH_3)CH_2C_6H_5$
(>20)	**18** = $C_{12}H_{25}$



(0.8)	**19** R = C_2H_5
(20.0)	**20** R = C_5H_{11}

(0.0003)	**docetaxel**
(0.1 - 0.2)	**doxorubicine**

Figure 1. Structures of gossypol derivatives and IC_{50} on KB cells (μM).

of gossypol or gossypolone on the growth of these cells in the presence of radical scavengers.

2. Chemistry

The toxicity of gossypol has been associated by many authors with the presence of the aldehyde groups which can easily bind nucleophilic targets of biological importance.

These considerations prompted us to synthesize new gossypol derivatives; Schiff's bases of gossypol; reduced gossypol; Schiff's bases of gossypolone and to compare their cytotoxicity.

The protected tetra and hexamethoxy ethers of gossypol have also been synthesized to evaluate the toxicity associated with the phenolic groups in gossypol.

For structure–activity comparison we reduced the aldehyde groups into alcohol using a selective reducing agent.

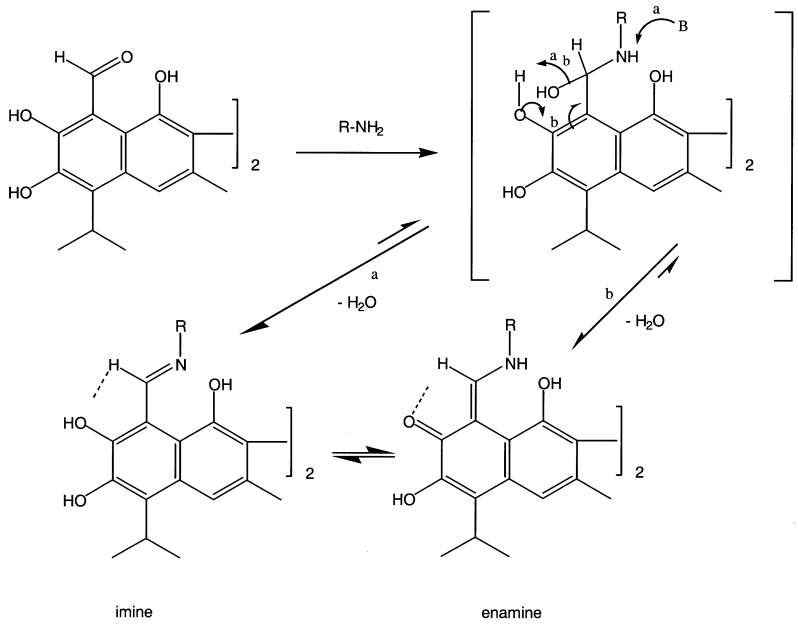


Figure 2.

One should note that gossypol exhibits atropisomerism due to hindered rotation about the binaphthyl bond. Since the gossypol used in this work was racemic and none of the derivatives were resolved, the differences in cytotoxic properties of their (+) and (–) isomers were not determined.

2.1. Coupling with amines – Schiff's bases of gossypol

Many Schiff's bases resulting from the coupling to amines of racemic gossypol or its enantiomers have already been described [8, 20, 23, 24]. Biological activities of the compounds are dependent both on the structure of the Schiff's base and also on the hydrophobicity of the substituting chain [20].

The high reactivity of the aldehyde groups in gossypol or in gossypolone enables the rapid coupling of nucleophiles followed by a possible dehydration of an intermediate carbinol (*figure 2*). The molecular structure of the Schiff's bases depends on the equilibrium between the imino and the enamine form and the enamine proton has been used as a probe for the NMR quantitation of the equilibrium [25]. Since NMR studies are often performed in aprotic solvents, the position of the observed equilibrium is not representative of the equilibrium in a physiological medium [26]. Thus NMR data could scarcely correlate with the biological activities, and the sharp structure–activity relationships observed for the antitumour activity [8] do not reflect the position of the enamine–imine equilibrium observed in aprotic solvents.

Schiff's bases were synthesized by reacting gossypol (compounds **6**–**18**) or gossypolone (compounds **19**–**20**) to the amines in a mixture of ether/isopropanol under mild conditions (25–45 °C, argon). After 5–30 min the product precipitated and could be recrystallized except for the methylamine derivative which was insoluble in the common solvents and which was prepared from a solution in dimethylformamide. NMR studies of these compounds in deuteriochloroform revealed the enamine structure for compounds **7**, **10**, **12**, **16** and **17**.

2.2. Reduction of gossypol

For the reduction of gossypol derivatives, different approaches have been proposed. Reduction of gossypol hexamethoxy derivative by hydrogen and platinum in acetic acid was attempted for the first time in 1930 by Adams et al. [27]. They obtained the deoxygossypol tetramethyl ether. A direct reduction of gossypol with $LiAlH_4$ followed by acetylation of the reaction mixture led to deoxygossypol tetraacetate and methylapogossypol hexaacetate [28].

More recently, Deck et al. reduced gossypol 1,1′ dimethyl ether with $NaBH_4$ and they obtained the corresponding alcohol [29].

Using $NaBH_3CN$, a smooth reducing agent, selective of the aldehyde group [30], we were able to obtain directly the alcohol from gossypol itself. The alcohol was purified by HPLC.

3. Results and discussion

The following new derivatives of gossypol have been synthesized and characterized: gossypolol **3** and imines of gossypolone **19** and **20**. Imines of gossypol **6–18** have been fully characterized (MS, NMR) and gossypol tetramethoxy ether **4** has been attributed a structure which is different from that previously proposed [1], considering its reactivity and NMR data.

The cytotoxic activities of compounds **1–20** are reported in *figure 1*. We found the ethylamine derivative **7** more toxic than other Schiff's bases of gossypol against KB cells (IC_{50} 5.6 μM), immediately followed by **8**, **9** and **11** (6.5, 6.6 and 6.7 μM, respectively).

Liang et al. [8] have determined the antiproliferative activities of **1**, **2**, **8**, **9**, **10** on MCF7, MCF7/adr malignant cells and HBL-100 immortalized human mammary epithelial cells. They concluded that, among numerous other Schiff's bases of gossypol, the isopropyl amine derivative **9**, was the most active.

We found racemic gossypolone **2** to be more toxic (2.4 μM) than gossypol and any gossypol Schiff's base. Interestingly enough, the Schiff's base of gossypolone **19** was the most toxic derivative (0.8 μM), whereas gossypolol **3** was almost inactive as well as methoxy-protected gossypol derivatives **4** and **5**.

Our results on KB cells parallel the results obtained by Liang et al. [8] on the antiproliferative effects of racemic gossypolone and Schiff's bases of gossypol. From the other reports in the literature it is difficult to draw firm conclusions since cytotoxicity was measured using different methods and on different cell lines. For instance, Shelley et al. reported recently that racemic gossypol and racemic gossypolone displayed IC_{50} values ranging from 20–30 μM [31]. Blackstaffe et al. reported IC_{50} values in an MTT viability assay of 22 μM for gossypol and gossypolone on the SK-mel-19 cell line and 22 μM for gossypol and no toxicity for gossypolone on the SK-mel-28 cell line [22].

Our data confirm that the cytotoxicity of gossypol derivatives depends on the availability of the phenolic oxygen atoms (**4** and **5** are inactive) and also on the bulk and structure of the radical substituent in a Schiff's base. In addition, the oxidation state of gossypol seems to play an important role since gossypolone and gossypolone Schiff's bases are more active than the reduced parent compound.

This last fact prompted us to evaluate the idea that gossypolone, which is a major metabolite of gossypol, could be, to a large extent, the toxic agent for the cells. Following this hypothesis, co-incubation of antioxidants or radical scavengers should reduce the toxicity of gossypol and less that of gossypolone.

For these experiments it was necessary to eliminate any compound known to bind gossypol or protect it from oxidation in the culture medium [32]. Furthermore, in view of the potential in vivo antitumour activities of gossypol and its derivatives, it was of interest to determine whether or not the various constituents of serum had an influence on their cytotoxic activities in vitro.

Table I shows that when gossypol and gossypolone are tested for their cytotoxic effects on KB cell monolayers maintained either in serum-free medium or in medium containing 10% foetal calf serum, the IC_{50} values of the compounds are 5–10 times lower in the serum-free medium. In addition, the toxicity of gossypol is more affected by the presence of serum than that of gossypolone.

Among the possible explanations for the inhibitory effect of serum on the activities of both compounds, we examined the role which free radical scavengers, known to be present in serum, could exert. To that effect, catalase, mannitol, glutathione and vitamin E (α-tocopherol) were added to KB cell cultures at non-

Table I. Effect of foetal calf serum on the cytotoxic activities of gossypol and gossypolone.

Medium containing 10 % FCS					
Gossypol or gossypolone concentration (μM)	10	5	2.5	1	0.5
% cell destruction with gossypol	60	50	30	0	0
% cell destruction with gossypolone	85	68	60	42	21
IC_{50} gossypol = 5 μM; IC_{50} gossypolone = 1.2 μM					
Medium without FCS					
Gossypol or gossypolone concentration (μM)	10	5	2.5	1	0.5
% cell destruction with gossypol	100	100	91	86	75
% cell destruction with gossypolone	91	80	74	68	58
IC_{50} of gossypol or gossypolone < 0.5 μM					

Table II. Effect of free radical scavengers on the cytotoxic activities of gossypol and gossypolone.

Treatment	Cell mortality after 48 h incubation (%)
Gossypol 0.5 μM	63
Gossypol 0.5 μM + catalase (150 μg/mL)	31
Gossypol 0.5 μM + mannitol (25 mM)	37
Gossypol 0.5 μM + glutathione (5 mM)	57
Gossypolone 0.5 μM	49
Gossypolone 0.5 μM + catalase (150 μg/mL)	27
Gossypolone 0.5 μM + mannitol (25 mM)	20
Gossypolone 0.5 μM + glutathione (5 mM)	72

cytotoxic concentrations and at the same time as gossypol and gossypolone. *Table II* shows that, under such conditions, catalase and mannitol (both known to interact with H_2O_2 formation) did exert a significant inhibitory effect on the cytotoxic activities of gossypol and gossypolone. Glutathione had no effect and results with α-tocopherol were inconsistent. Again, the toxicity of gossypol remains higher than the toxicity of gossypolone in the presence of radical scavengers, suggesting that serum actually protects the cells from gossypol not only by quenching free radicals.

A preliminary clinical study of gossypol in advanced human cancer has been performed by Stein et al. [33]. This was essentially a phase I study in which 34 patients were given escalating doses of oral gossypol. No major adverse effects occurred and there was no evidence of haematological or biochemical disturbances, the dose-limiting toxicity being emesis. There was no evidence either of tumour regression, but in our opinion these negative results do not rule out the possibility that gossypol derivatives endowed with higher in vitro cytotoxicity than that of gossypol itself and administered by various routes in combination with other anticancer agents could prove to play a useful role in the therapy of some cancer states.

Further studies are underway to synthesize new derivatives of gossypol and particularly of gossypol enantiomers which could, as pointed out by many authors [22, 31], lead to interesting antitumour drugs.

4. Experimental protocols

4.1. Chemistry

Reagents and solvents used for the reactions were purchased from Fluka. Gossypol-acetic acid **1** was extracted and purified following Carruth [21]. Mass spectra were determined at the Service de Spectrométrie de Masse de l'Institut de Chimie des Substances Naturelles (ICSN) on an AEI MS50 spectrometer or an MS80 RT Kratos spectrometer. ^{1}H-, ^{13}C-NMR spectra were recorded in $CDCl_3$ or DMSO-d_6 on a Bruker AM300 spectrometer. HPLC analysis was performed on a Waters HPLC 600E, with a reversed-phase Alltima C-18, 5 μm column (4.6 mm i.d. × 250 mm) by isocratic elution (I): 10% A, 90% B; (II): 20% A, 80% B the flow rate was 1 mL/min; (III): 2% A, 98% B the flow rate was 2 mL/min at 50 ± 1 °C, with the solvent system: A: 95% water, 5% CH_3CN, 0.1% TFA; B: 100% CH_3CN, 0.1% TFA. The detection wavelength was 254 nm.

4.1.1. Synthesis of 6,7,6′,7′-tetrahydroxy-5,5′-diisopropyl-3,3′-dimethyl-1,4,1′,4′-tetraoxo-1,4,1′,4′-tetrahydro-[2,2′]-binaphthalenyl-8,8′-dicarbaldehyde (gossypolone) **2**

Following the method described by Haas et al. [34]. A solution of 1.5 g (2.59 mmol) of **1** in 75 mL of acetone and 150 mL of acetic acid was heated on a steam bath (60–70 °C) during the addition of 112.5 mL of a 10% aqueous solution of ferric chloride hexahydrate (42 mmol) and for several minutes longer. The solution was cooled and 200 mL of water was added to precipitate a dark iron-containing compound which was removed and treated with a mixture of ether and aqueous 20% sulfuric acid. The liberated phenol was taken into the ether layer which was washed with water and dried (Na_2SO_4), and the ether was evaporated. The residue was precipitated from toluene/hexane to yield 0.6–0.7 g (42–49%). HPLC: K′(II): 2.75; EI-MS: m/z: 553 (M + Li); ^{1}H-NMR-$CDCl_3$: 1.46 (d, 12H, 2 HC–($\underline{CH_3}$)$_2$), 2.07 (s, 6H, 2 Ar–CH_3), 4.14 (m, 2H, 2 $\underline{HC}$–(CH_3)$_2$), 6.59 (s, 2H, 2 OH at 6,6′ positions), 10.60 (s, 2H, 2 CHO), 13.03 (s, 2H, 2 OH at 7,7′ positions); ^{13}C-NMR-$CDCl_3$:198.65 (CHO), 186.70, 184.60 (2 C=O at 1 and 4 positions).

4.1.2. Synthesis of 8,8′-bis-hydroxymethyl-5,5′-diisopropyl-3,3′-dimethyl-[2,2′]-binaphthalenyl-1,6,7,1′,6′,7′-hexaol (gossypolol or gossylic alcohol) **3**

A solution of 1 g (1.73 mmol) of **1** in 20 mL of methanol and 108 mg (1.73 mmol) of $NaBH_3CN$ and 2 N HCl/methanol was added dropwise with stirring to maintain the pH of the solution near 3–4. After ca. 5 h the methanol was evaporated at reduced pressure and the residue was taken up in 20 mL of water, saturated with sodium chloride and extracted with three 15 mL portions of ether, the combined extracts were dried (Na_2SO_4) and evaporated to give a residue (1 g), a small amount of the gossypol-acetic acid was detected in the residue by HPLC. To eliminate the gossypol-acetic acid, we used

Girard's T-reagent: 500 mg of the residue and 100 mg of Girard's T-reagent were dissolved in 50 mL of alcohol containing 10% acetic acid, the solution was heated for 30 min at 60 °C. Then, the reaction mixture was cooled, 50 mL of water was added, and solution was extracted with three 15 mL portions of ether. The organic phase was washed with water and dried over Na_2SO_4, the ether was removed in vacuo to yield 75 mg (15% yield) HPLC: k′(II): 3.04; EI-MS: m/z 545 (M + Na); ^{1}H-NMR-$CDCl_3$: 1.5 (m, 2 HC–($\underline{CH_3}$)$_2$), 2.2 (6H: 2 Ar–CH_3), 3.9 (2H: $\underline{HC}$–(CH_3)$_2$), 5.6 (4H: $\underline{CH_2}$OH); IR 3 517 cm^{-1} (alcohol).

4.1.3. Synthesis of 5,5′-diisopropyl-2,4,2′,4′-tetramethoxy-7,7′-dimethyl-2H,2′H-[8,8′]bi[naphtho[1,8-bc]furanyl]-3,3′-diol **4**

One gram (1.73 mmol) of **1** in a mixture solution of 5 mL of absolute methyl alcohol and 5 mL of dimethyl sulfate was stirred at room temperature under argon. When the solid was completely dissolved, 7 mL of 10% methyl alcoholic potassium hydroxide was added dropwise and the mixture was allowed to stand for 24 h at room temperature. The solution turned a deep brown colour and a precipitate of potassium sulfate formed, the excess dimethyl sulfate was decomposed with 10% aqueous sodium hydroxide solution. The white flocculent precipitate that formed was filtered, washed with cool methanol and dried to yield 0.85 g (85.5% yield). HPLC: k′(I): 2.83; EI-MS: m/z: 575 (MH$^+$); ^{1}H-NMR-$CDCl_3$: 1.52 (d, 12H, 2 HC–($\underline{CH_3}$)$_2$), 2.29 (s, 6H, 2 Ar–CH_3), 3.83 (m, 2H, 2 $\underline{HC}$–(CH_3)$_2$), 3.28 (s, 6H, 2 O–HC–O$\underline{CH_3}$), 4.19 (s, 6H, 2 O$\underline{CH_3}$ at positions 6,6′), 6.28 (s, 2H, 2 OH at 7,7′ positions), 7.1 (s, 2H, 2 O–$\underline{H}$C–OAr), 7.42 (s, 2H, 2 Ar–H). These data, compared to NMR data from Haas et al. [34], are more consistent with a methoxy group in positions 6,6′ than in position 7,7′ as previously proposed by Adams et al. [1].

4.1.4. Synthesis of 5,5′-diisopropyl-2,3,4,2′,3′,4′-hexamethoxy-7,7′-dimethyl-2H,2′H-[8,8′]bi[naphtho[1,8-bc]furanyl] **5**

Two grams (3.46 mmol) of **1** in a solution of 20 mL of absolute methyl alcohol and 20 mL of dimethyl sulfate was stirred at room temperature under argon. When solution was complete, a solution of 30 mL 10% methyl alcoholic potassium hydroxide was added dropwise and the mixture was allowed to stir for 48 h at room temperature. Excess dimethyl sulfate was decomposed with 10% aqueous sodium hydroxide solution. The white precipitate that formed was filtered, washed with cool methanol and dried: 1.6 g (76.9% yield). HPLC: k′(I): 5.31 and 5.85; EI-MS: m/z: 603 (MH$^+$); ^{1}H-NMR-$CDCl_3$ (300 MHz) 1.54 (d, 12H, 2 HC–($\underline{CH_3}$)$_2$), 2.32 (m, 6H, 2 Ar–CH_3), 3.31–3.46 (m, 6H, 2 O–HC–O$\underline{CH_3}$), 3.83–4.19 (m, 14H, 2 $\underline{HC}$–(CH_3)$_2$, 4 OCH_3 at 6,6′,7,7′ positions), 6.95–7.06 (m, 2H, 2 O–$\underline{H}$C–OAr), 7.48 (d, 2H, 2 Ar-H).

4.1.5. General method for the synthesis of gossypol and gossypolone Schiff's bases: **6–18** *and* **19**, **20**

Two hundred milligrams (0.35 mmol) of **1** [(0.37 mmol) of **2**] was dissolved in a solution of 5 mL ether and 2 mL of isopropanol and stirred at room temperature. The amine (1.4 mmol) was added and heated for 5 min at 45 °C and the mixture was allowed to stand, under argon, for 2 h at room temperature. The precipitate was filtered off, washed with hexane and vacuum dried to give the Schiff's base as a yellow solid.

6: 5,5′-diisopropyl-3,3′-dimethyl-8,8′-bis-methyliminomethyl-[2,2′]binaphthalenyl-1,6,7,1′,6′,7′-hexaol. The precipitate, 180.4 mg (yield 95%) was obtained from a solution of methylamine in dimethylformamide. It was purified by repeated precipitations from dimethylformamide solutions The very low solubility of this compound in chloroform did not allow NMR analysis or mass spectrometry.

7: 8,8′-bis-ethyliminomethyl-5,5′-diisopropyl-3,3′-dimethyl-[2,2′]binaphthalenyl-1,6,7,1′,6′,7′-hexaol: see general method for synthesis. Obtained 190 mg (yield 96%) HPLC: k′(III): 0.71, EI-MS: m/z 573 (MH$^+$); ^{1}H-NMR-$CDCl_3$:1.37 (t, 6H, 2 HC=N–CH_2–$\underline{CH_3}$), 1.52 (d, 12H, 2 HC-($\underline{CH_3}$)$_2$), 2.12 (s, 6H, 2 Ar–CH_3), 3.52 (m, 4H, 2 HC=N–$\underline{CH_2}$–CH_3), 3.74 (m, 2H, 2 $\underline{HC}$–(CH_3)$_2$), 7.59 (s, 2H, 2 Ar–H), 9.69 (d, 2H, 2 HC=N–R); 13.34 (br, 2H, 2 OH at 7,7′ positions).

8: 5,5′-diisopropyl-3,3′-dimethyl-8,8′-bis-propyliminomethyl-[2,2′]binaphthalenyl-1,6,7,1′,6′,7′-hexaol: see general method for synthesis. Obtained 191 mg (yield 92%) HPLC: k′(III): 1.09; EI-MS: m/z 601 (MH$^+$); ^{1}H-NMR-$CDCl_3$: 1.02 (t, 6H, 2 HC=N–CH_2–CH_2–$\underline{CH_3}$), 1.54 (d, 12H, 2 HC–($\underline{CH_3}$)$_2$), 1.73 (m, 4H, 2 HC=N–CH_2–$\underline{CH_2}$–CH_3), 2.13 (s, 6H, 2 Ar–CH_3), 3.46 (t, 4H, 2 HC=N–$\underline{CH_2}$–CH_2–CH_3), 3.74 (m, 2H, 2 $\underline{HC}$–(CH_3)$_2$), 7.60 (s, 2H, 2 Ar–H), 9.65 (d, 2H, 2 HC=N–R); 13.27 (br, 2H, 2 OH at 7,7′ positions).

9: 5,5′-diisopropyl-8,8′-bis-(isopropylimino-methyl)-3,3′-dimethyl-[2,2′]binaphthalenyl-1,6,7,1′,6′,7′-hexaol: see general method for synthesis. Obtained 201 mg (yield 96%). HPLC: k′(III): 1.04; EI-MS: m/z 601 (MH$^+$); ^{1}H-NMR-$CDCl_3$: 1.39 (t, 6H, 2 HC=N–CH–($\underline{CH_3}$)$_2$), 1.55 (d, 12H, 2 HC–($\underline{CH_3}$)$_2$), 2.13 (s, 6H, 2 Ar–CH_3), 3.46 (t, 4H, 2 HC=N–$\underline{CH_2}$–CH_2–CH_3), 3.73 (m, 4H, 2 HC=N–$\underline{CH}$–(CH_3)$_2$) and 2 $\underline{HC}$–(CH_3)$_2$), 7.61 (s, 2H, 2 Ar–H), 9.72 (d, 2H, 2 HC=N–R); 13.28 (br, 2H, 2 OH at 7,7′ positions).

10: 8,8′-bis-butyliminomethyl-5,5′-diisopropyl-3,3′-dimethyl-[2,2′]binaphthalenyl-1,6,7,1′,6′,7′-hexaol: see general method for synthesis. Obtained 196 mg (yield 90%) HPLC: k′(III): 1.58; EI-MS: m/z 635 (M + Li), 629 (MH^+); ^{1}H-NMR-$CDCl_3$: 0.98 (t, 6H, 2 HC=N–CH_2–CH_2–CH_2–$\underline{CH_3}$), 1.43 (m, 4H, 2 HC=N–CH_2–CH_2–$\underline{CH_2}$–CH_3),1.53 (d, 12H, 2 HC–$(\underline{CH_3})_2$),1.72 (m, 4H, 2 HC=N–CH_2–$\underline{CH_2}$–CH_2–CH_3), 2.13 (s, 6H, 2 Ar–CH_3), 3.49 (t, 4H, 2 HC=N–$\underline{CH_2}$–CH_2–CH_2–CH_3), δ 3.74 (m, 2H, 2 $\underline{HC}$–$(CH_3)_2$), 7.61 (s, 2H, 2 Ar–H), 9.65 (s, 2H, 2 HC=N–R), 13.35 (br, 2H, 2 OH at 7,7′ positions).

11: 8,8′-bis-(sec-butylimino-methyl)-5,5′-diisopropyl-3,3′-dimethyl-[2,2′]binaphthalenyl-1,6,7,1′,6′,7′-hexaol: see general method for synthesis. Obtained 196 mg (yield 90%) HPLC: k′(III): 1.51; EI-MS:m/z 635 (M + Li), 629 (MH^+); ^{1}H-NMR-$CDCl_3$: 0.98–1.38 (m, 12H, 2 CH_2–CH–$(\underline{CH_3})_2$), 1.54 (d, 12H, 2 CH–$(\underline{CH_3})_2$), 1.67 (m, 4H, 2 $\underline{CH_2}$–CH–$(CH_3)_2$), 2.12 (s, 6H, 2 Ar–$\underline{CH_3}$), 3.44 (m, 2H, 2 CH_2–$\underline{CH}$–$(CH_3)_2$) 3.76 (m, 2H, 2 $\underline{CH}$–$(CH_3)_2$), 5.58 (s, 2H, 2 OH at 1,1′ positions), 7.61 (s, 2H, 2 Ar–H), 8.03 (s, 2H, 2 OH at 6,6′ positions), 9.7 (2s, 2H, 2 HC=N–R), 13.3 (br, 2H, 2 OH at 7,7′ positions).

12: 8,8′-bis-(tert-butylimino-methyl)-5,5′-diisopropyl-3,3′-dimethyl-[2,2′]binaphthalenyl-1,6,7,1′,6′,7′-hexaol: see general method for synthesis. Obtained 190 mg (yield 87%) HPLC: k′(III): 0.77, 1.42; EI-MS: m/z 635 (M + Li), 629 (MH^+); ^{1}H-NMR-$CDCl_3$: 1.27 (s, C–$(\underline{CH_3})_3$), 1.53 (d, 12H, 2 CH–$(\underline{CH_3})_2$), 2.13 (s, 6H, 2 Ar–$\underline{CH_3}$), 3.74 (m, 2 $\underline{CH}$–$(CH_3)_2$), 7.62 (s, 2H, 2 Ar–H), 8.02 (br, 2H, 2 OH at 6,6′ positions), 9.84 (2s, 2H, 2 HC=N–R), 13.43 (br, 2H, 2 OH at 7,7′ positions).

13: 5,5′-diisopropyl-3,3′-dimethyl-8,8′-bis-pentyl-iminomethyl-[2,2′]binaphthalenyl-1,6,7,1′,6′,7′-hexaol: see general method for synthesis. Obtained 190 mg (yield 83%). HPLC: k′(III): 2.31; EI-MS: m/z 663 (M + Li), 657 (MH^+); ^{1}H-NMR-$CDCl_3$: 0.91 (t, 6H, 2 HC=N–$(CH_2)_4$–$\underline{CH_3}$), 1.34 (m, 8H, 2 HC=N–$(CH_2)_3$–$\underline{CH_2}$–CH_3, 2 HC=N–$(CH_2)_2$–$\underline{CH_2}$–CH_2–CH_3), 1.53 (d, 12H, 2 HC–$(\underline{CH_3})_2$), δ 1.71 (m, 4H, 2 HC=N–CH_2–$\underline{CH_2}$–$(CH_2)_2$–CH_3), δ 2.13 (s, 6H, 2 Ar–CH_3), δ 3.49 (t, 4H, 2 HC=N–$\underline{CH_2}$–$(CH_2)_3$–CH_3), δ 3.74 (m, 2H, 2 $\underline{HC}$–$(CH_3)_2$), δ 7.61 (s, 2H, 2 Ar–H), 9.64 (s, 2H, 2 HC=N–R); 13.31 (br, 2H, 2 OH at 7,7′ positions).

14: 8,8′-bis-hexyliminomethyl-5,5′-diisopropyl-3,3′-dimethyl-[2,2′]binaphthalenyl-1,6,7,1′,6′,7′-hexaol: see general method for synthesis. Obtained 204 mg (yield 86%) HPLC: k′(III): 3.43; EI-MS: m/z 691 (M + Li), 684 (M^+); ^{1}H-NMR-$CDCl_3$: 0.87 (t, 6H, 2 HC=N–$(CH_2)_5$–$\underline{CH_3}$), 1.28 (m, 12H, 2 HC=N–$(CH_2)_4$–$\underline{CH_2}$–CH_3, 2 HC=N–$(CH_2)_3$–$\underline{CH_2}$–CH_2–CH_3, 2 HC=N–$(CH_2)_2$–$\underline{CH_2}$–$(CH_2)_2$–CH_3), δ 1.55 (d, 12H, 2 HC–$(\underline{CH_3})_2$), δ 1.73 (m, 4H, 2 HC=N–CH_2–$\underline{CH_2}$–$(CH_2)_3$–CH_3), 2.13 (s, 6H, 2 Ar–CH_3), δ 3.49 (t, 4H, 2 HC=N–$\underline{CH_2}$–$(CH_2)_4$–CH_3), 3.74 (m, 2H, 2 HC–$(CH_3)_2$), 7.61 (s, 2H, 2 Ar–H), δ 9.64 (s, 2H, 2 HC=N–R); 13.31 (br, 2H, 2 OH at 7,7′ positions).

15: 8,8′-bis-heptyliminomethyl-5,5′-diisopropyl-3,3′-dimethyl-[2,2′]binaphthalenyl-1,6,7,1′,6′,7′-hexaol: see general method for synthesis. Obtained 176 mg (yield 71%) HPLC: k′(III): 5.23; EI-MS: m/z 713 (MH^+); ^{1}H-NMR-$CDCl_3$: 0.88 (t, 6H, 2 HC=N–$(CH_2)_6$–$\underline{CH_3}$), 1.31 (m, 18H, 2 HC=N–$(CH_2)_5$–$\underline{CH_2}$–CH_3, 2 HC=N–$(CH_2)_4$–$\underline{CH_2}$–CH_2–CH_3, 2 HC=N–$(CH_2)_3$–$\underline{CH_2}$–$(CH_2)_2$–CH_3, 2 HC=N–$(CH_2)_2$–$\underline{CH_2}$–$(CH_2)_3$–CH_3), 1.54 (d, 12H, 2 HC–$(\underline{CH_3})_2$), 1.71 (m, 4H, 2 HC=N–CH_2–$\underline{CH_2}$–$(CH_2)_4$–CH_3), 2.13 (s, 6H, 2 Ar–CH_3), 3.49 (t, 4H, 2 HC=N–$\underline{CH_2}$–$(CH_2)_5$–CH_3), 3.74 (m, 2H, 2 HC–$(CH_3)_2$), 7.61 (s, 2H, 2 Ar–H), 9.65 (s, 2H, 2 $\underline{HC}$=N–R);13.34 (br, 2H, 2 OH at 7,7′ positions).

16: 5,5′-diisopropyl-3,3′-dimethyl-8,8′-bis[(2-phenyl-propylimino)-methyl]-[2,2′]binaphthalenyl-1,6,7,1′,6′,7′-hexaol: see general method for synthesis. Obtained 241 mg (yield 92%) HPLC: k′(III): 1.00, 1.57; EI-MS: m/z 753 (MH^+); ^{1}H-NMR-$CDCl_3$: 1.4 (d, 6H, 2 CH_2–CH($\underline{CH_3}$)–C_6H_5), 1.53 (d, 12H, 2 HC–$(\underline{CH_3})_2$), 2.1 (s, 6H, 2 Ar–CH_3), 3.08 (m, 2H, 2 CH_2–$\underline{CH}(CH_3)$–C_6H_5), 3.67 (m, 6H, 2 $\underline{CH_2}$–$CH(CH_3)$–C_6H_5 and 2 HC–$(\underline{CH_3})_2$), 5.51 (br, 2H, 2 OH at 1,1′ positions), 7.31 (m, 10H, 2 CH_2–$CH(CH_3)$–$\underline{C_6H_5}$), 7.59 (s, 2H, 2 Ar–H), 7.99 (br, 2H, 2 OH at 6,6′ positions), 9.5 (d, 2H, 2 HC=N–R), 13.32 (br, 2H, 2 OH at 7,7′ positions).

17: 2-({1,6,7,1′,6′,7′-hexahydroxy-5,5′-diisopropyl-8′-[(1-methoxycarbonyl-2-phenyl-ethylimino)-methyl]-3,3′-dimethyl-[2,2′]binaphthalenyl-8-ylmethylene}-amino)-3-phenyl-propionic acid methyl ester: see general method for synthesis. Obtained 220 mg (yield 84%). EI-MS: m/z 753 (MH^+); ^{1}H-NMR-$CDCl_3$: 1.53 (d, 12H, 2 HC–$(\underline{CH_3})_2$), 2.07 (s, 6H, 2 Ar–CH_3), 3.17–3.32 (m, 4H, 2 CH($COOCH_3$)$\underline{CH_2}$–C_6H_5), 3.72 (m, 2H,2 HC–$(\underline{CH_3})_2$), 3.77 (s, 6H, 2 CH($COO\underline{CH_3}$)CH_2–C_6H_5), 4.3 (m, 2H, 2 $\underline{CH}$($COOCH_3$)CH_2–C_6H_5), 5.37 (br, 2H, 2 OH at 1,1′ positions), 7.23 (m, 10H, 2 CH($COOCH_3$)CH_2–$\underline{C_6H_5}$), 7.55 (s, 2H, 2 Ar–H), 7.94 (s, 2H, 2 OH at 6,6′ positions), 9.27–9.39 (d, 2H, 2 HC=N–R), 13.36 (br, 2H, 2 OH at 7,7′ positions).

18: 8,8′-bis-dodecyliminomethyl-5,5′-diisopropyl-3,3′-dimethyl-[2,2′]binaphthalenyl-1,6,7,1′,6′,7′-hexaol: see general method for synthesis. Obtained 270 mg (yield 91%) HPLC: k′(III): EI-MS: m/z 853 (M^+); ^{1}H-NMR-$CDCl_3$: 0.88 (t, 6H, 2 HC=N–$(CH_2)_{11}$–$\underline{CH_3}$), 1.27 (m, 36H, 2 HC=N–$(CH_2)_2$–$(\underline{CH_2})_9$–CH_3), 1.53 (d, 12H, 2 HC–$(\underline{CH_3})_2$), 1.70 (m, 4H, 2 HC=N–CH_2–

$\underline{CH_2}$–$(CH_2)_9$–CH_3), 2.13 (s, 6H, 2 Ar–CH_3), 3.48 (t, 4H, 2 HC=N–$\underline{CH_2}$–$(CH_2)_{10}$–CH_3), δ 3.74 (m, 2H, 2 $\underline{HC}$–$(CH_3)_2$), δ 7.61 (s, 2H, 2 Ar–H), 9.65 (s, 2H, 2 HC=N–R); 13.34 (br, 2H, 2 OH at 7,7′ positions).

19: 8,8′-bis-ethyliminomethyl-6,7,6′,7′-tetrahydroxy-5,5′-diisopropyl-3,3′-dimethyl-[2,2′]binaphthalenyl-1,4,1′,4′-tetraone: see general method for synthesis. Obtained 182 mg (yield 82%) HPLC: k′(III): EI-MS: m/z 601 (MH^+); ^{1}H-NMR-$CDCl_3$: 1.27–1.44 (m, 18H, 2 HC–$(\underline{CH_3})_2$, 2 HC=N–CH_2–$\underline{CH_3}$), 1.99 (s, 6H, 2 Ar–$\underline{CH_3}$), 3.69 (q, 4H, 2 HC=N–$\underline{CH_2}$–CH_3), 3.85 (m, 2H, 2 $\underline{HC}$–$(CH_3)_2$), δ 9.64 (d, 2H, 2 HC=N–R).

20: 6,7,6′,7′-tetrahydroxy-5,5′-diisopropyl-3,3′-dimethyl-8,8′-bis-pentyliminomethyl-[2,2′]binaphthalenyl-1,4,1′,4′-tetraone: see general method for synthesis. Obtained 176 mg (yield 70%) HPLC: k′(III): EI-MS: m/z 685 (MH^+); ^{1}H-NMR-$CDCl_3$: 0.91 (t, 6H, 2 HC=N–CH_2–$\underline{CH_3}$), 1.35–1.46 (20H, 2 HC–$(\underline{CH_3})_2$, 2 HC=N–$(CH_2)_2$–$(\underline{CH_2})_2$–CH_3), 1.75 (m, 4H, 2 HC=N–CH_2–$\underline{CH_2}$–$(CH_2)_2$–CH_3), 1.99 (s, 6H, 2 Ar–CH_3), 3.60 (t, 4H, 2 HC=N–$\underline{CH_2}$–$(CH_2)_3$–CH_3), 3.87 (m, 2H, 2 HC–$(CH_3)_2$), 9.60 (d, 2H, 2 HC=N–R).

4.2. Biological studies

4.2.1. Cytotoxicity

The in vitro cytotoxic activities of all the various gossypol derivatives were evaluated in KB cells (human epidermoid carcinoma of the mouth), originally obtained from the American Type Culture Collection.

The KB cell line was grown in MEM (minimal essential medium, with Earle's salt solution), purchased at Seromed, containing 10% foetal calf serum, 2 mM L-glutamine, 60 u/ml penicillin G and streptomycin sulfate and 40 μg/mL gentamycin. For the tests, KB cells were grown as monolayers in Nunc 24-well plastic plates (25 000 cells seeded per well in 1 mL of medium).

The compounds were dissolved in dimethylsulfoxide (DMSO), serial dilutions were made in this solvent and added to the cultures in a volume of 10 μL per well, immediately after plating the cells (final concentration of DMSO < 1%). Control cultures received an equivalent dilution of DMSO. All cultures were incubated at 37 °C in a 95% air/5%CO_2 humidified incubator. After 3 days incubation cell viability in the monolayer was determined by incubation for a further 8–16 h after adding 100 μL of a 0.02% solution in medium of the vital dye neutral red to each well, followed by washing with phosphate buffered saline, lysis of the cells with a 1% solution of sodium lauryl-dodecyl sulfate and photometric quantification of the extracted dye at 540 nm, using a Uniskam-II microplate reader (Labosystems, Life Sciences International).

For most of the compounds examined, final concentrations tested ranged from 0.5–10 μM and each concentration was added to two wells per plastic plate. The IC_{50} of each compound was calculated by plotting the percent of cell death against the concentration of the test compound.

For the purpose of comparison, the cytotoxic activities of two clinically useful anticancer agents, doxorubicin and docetaxel, were determined using the same method as described above. Doxorubicin was purchased as an injectable solution for hospital use, docetaxel was a gift from Rhône-Poulenc Rorer.

The possible implication of free radical formation in the cytotoxic activities of gossypol and gossypolone was examined by studying the effect of various scavengers on these activities. Inasmuch as calf serum present in the culture medium does contain endogenous scavengers of free radicals, these tests were performed on cells maintained in serum-free medium.

KB cells were first grown as usual in the presence of foetal calf serum, but after 24 hours incubation at 37 °C adherent cell layers in the wells were washed with PBS and the medium was changed to serum-free MEM. Mannitol, catalase and α-tocopherol (all purchased from Sigma) were added to the cultures at predetermined non-toxic concentrations, at the same time as various concentrations of gossypol and gossypolone. The percent of cell destruction was determined after 48 h incubation at 37 °C by the neutral red viability test as described above.

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