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Short communication

Cytotoxicity of enantiomers of gossypol Schiff's bases and optical stability of gossypolone

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Abstract

Optical Schiff's bases of gossypol were prepared with chiral gossypol and ethylamine. As has been similarly observed among the gossypol enantiomers, the (–)-gossypol ethylimine was more active than either the (+)-gossypol ethylimine or the racemic gossypol ethylimine against KB and MCF7 cells. Gossypolone was also observed to be more toxic than gossypol against both cell lines. All of the gossypol products tested showed comparable toxicity toward MCF7/ADR (adriblastine-resistant) cells. Attempts at producing chiral gossypolone from chiral gossypol failed because of rapid racemization. In addition, the Schiff's base derivatives of gossypolone formed with *R*-(+)-2-amino-3-phenyl-1-propanol could only be separated at reduced temperature, indicating that gossypolone Schiff's bases are less optical stable than gossypol Schiff's bases.

Keywords: Gossypol; Gossypolone; Enantiomers; KB; MCF-7; MCF-7/ADR; Multi-drug resistance

1. Introduction

Gossypol (Fig. 1), a binaphthyl pigment present in the cotton plant and a number of other members of the Malvaceae family, is chiral because of steric hindrance to rotation about the internaphthyl bond [1]. Gossypol exhibits a number of interesting types of biological activity, including antifertility [2,3], anti-malarial [4,5], anti-HIV [6,7] effects. More recently, gossypol has been shown to exhibit toxicity towards cancer cells [8–11].

In a number of these studies, the (–)-enantiomer of gossypol has been shown to be substantially more active than the (+)-enantiomer [3,9,10,12–14]. To date, most studies on gossypol derivatives have focused on the biological activities of racemic products. The cytotoxic potential of chiral gossypol derivatives has not been evaluated, despite the higher activities of (–)-gossypol.

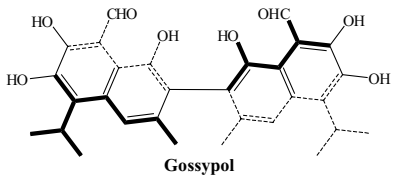

Fig. 1. Structure of gossypol.

In a previous paper, we showed that some Schiff's bases of racemic gossypol exhibited higher toxicities than gossypol itself [15]. In this work, we report on the synthesis of Schiff's bases of chiral gossypol and on the relative toxicities of these compounds against KB cells (mouth epidermoid carcinoma cells), MCF-7 cells (breast carcinoma cells), and MCF-7/ADR cells (doxorubicin resistant breast carcinoma cells).

Because gossypolone has also been shown to be active against cancer cell lines [15] we also attempted to extend the study to the gossypolone enantiomers and chiral derivatives. These compounds, however, racemized very quickly at room temperature, and it was not possible to test their activity.

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2. Results and discussion

2.1. Synthesis and cytotoxicity of Schiff's bases of gossypol enantiomers

Gossypol enantiomers were prepared from racemic gossypol acetic acid by the low-temperature crystallization of the conglomerate gossypol/acetone (1:3) crystal form [16]. The final preparations were >99% chemically and optically pure and yielded CD curves (Fig. 2**A**) identical to those previously reported for (+)- and (–)-gossypol [17].

Schiff's bases of both gossypol enantiomers were synthesized with ethylamine by previously described methods [15]. Ethylamine derivatives were used in this work because racemic gossypol–ethylamine had higher toxicity against KB cells than did racemic gossypol [15]. CD curves of the ethylamine derivatives showed that the formation of the bases did not promote racemization of the gossypol backbone (Fig. 2**B**). The biological activity of the new compounds was screened by measuring their in vitro antiproliferative effects against malignant KB cells, wild-type MCF-7/WT cells, and doxorubicin resistant MCF-7/ADR cells (Table 1).

In all three cell lines, we confirmed our previous result that the Schiff's base of (±)-gossypol with ethylamine was more toxic than (±)-gossypol itself (Table 1). In addition, the higher toxicity of (–)-gossypol over (+)-gossypol was also observed. The chiral ethylamine derivatives showed a similar trend, with the (–)-enantiomer exhibiting higher activity that the (+)-enantiomer, except for the MCF-7/ADR cells, where the opposite trend was observed. The toxicity of the chiral ethylamine gossypol derivatives was similar to that of gossypolone or the gossypolone Schiff's bases.

In MCF7/ADR and MCF7/WT cells, (–)-gossypol appeared to be almost an order of magnitude more toxic than (+)-gossypol. The enhanced toxicity of (–)-gossypol in different biological preparations [2,3,6] has been confirmed in our study and is in accordance with the results of Jaroszewski et al. [8], who used different methods to measure the toxicity of the gossypol enantiomers.

Table 1
IC_{50} values for (±)-, (+)-, and (–)-gossypol, the ethylimines of (±)- (+)- and (–)-gossypol, (±)-gossypolone and the methyl- and ethylimines of (±)-gossypolone

Compound	IC_{50} (μM)		
	KB	MCF-7/WT	MCF-7/ADR
(±)-Gossypol	1.80	1.60	1.30
(+)-Gossypol	9.50	3.60	4.40
(–)-Gossypol	1.20	0.35	0.26
Ethylimine of (±)-gossypol	1.00	0.45	0.40
Ethylimine of (+)-gossypol	1.60	0.99	0.28
Ethylimine of (–)-gossypol	0.30	0.20	0.80
(±)-Gossypolone	0.45	0.36	0.28
Methylimine of (±)-gossypolone	0.50	0.37	0.14
Ethylimine of (±)-gossypolone	0.55	0.29	0.35
Adriblastine		0.29	>30

Compared to the nonresistant cell line, MCF7/ADR cells exhibited a 100-fold increased resistance towards adriblastine, but little increased resistance towards the gossypol enantiomers and their derivatives. The toxicity of gossypol and its enantiomers on resistant cell lines has already been reported and attributed to the inhibition of certain GST isozymes and expression of hsp-70 [9] or the blocking of the P-170 (P-gp) pump [8]. Nevertheless, drug resistance is a complex mechanism where, among other factors, the overexpression of the efflux pump may be counterbalanced by the passive transport of the drug through the cytoplasmic membrane. Interestingly, the low resistance of MCF7/ADR cells for the gossypol ethylamine derivatives does not seem to

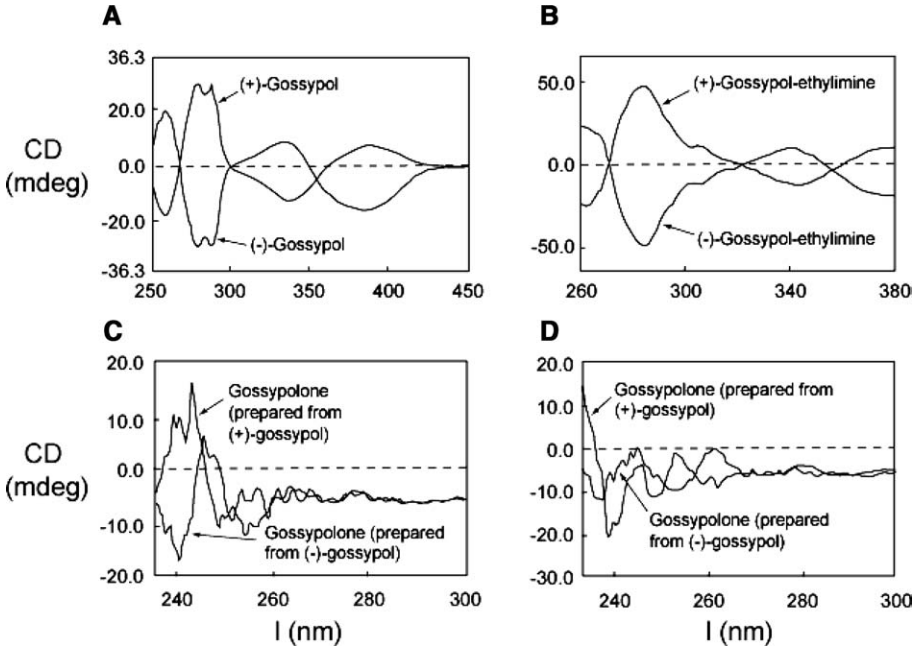

Fig. 2. CD curves for the gossypol enantiomers (**A**), (+)-gossypol ethylimine and (–)-gossypol ethylimine (**B**), chiral gossypolone enantiomers synthesized at room temperature (**C**), and chiral gossypolone enantiomers heated at 60 °C for 1 h (**D**).

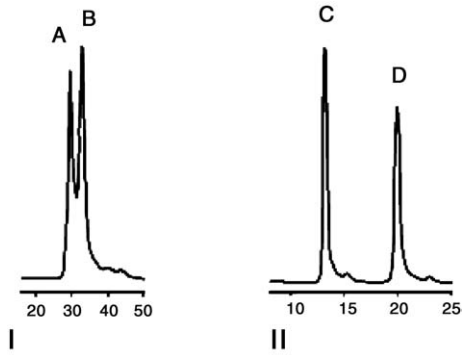

Fig. 3. HPLC separation of diastereomeric gossypol Schiff's bases prepared on a reverse-phase C8 column: *S*-(+)-2-aminobutane gossypol diastereomers (**I**) and *R*-(+)-2-amino-3-phenyl-1-propanol gossypol diastereomers (**II**).

depend strongly on the chirality of the gossypol backbone, but is more directly related to the derivatizing group of the gossypol imine.

In our assays, we found gossypolone to be as active against MCF-7/ADR cells as (–)-gossypol. In contrast, Liang et al. [18] found (–)-gossypol to be about three times more toxic than gossypolone against the same cell line. As discussed below, we could not separate the gossypolone enantiomers, but we observed that racemic gossypolone and its methylimine and ethylimine Schiff's bases displayed toxicities in the sub-micromolar range against all three cell lines.

2.2. Gossypolone enantiomers

Gossypolone and gossypolone Schiff's bases have been shown to be more toxic than the corresponding gossypol derivatives [15]. To prepare chiral gossypolone Schiff's bases, we first tried to prepare gossypolone enantiomers by oxidizing the individual gossypol enantiomers and treating these products with the amine to form the corresponding enantiomeric Schiff's bases. The method of Hass and Shirley [19] was used to perform the oxidation.

Starting from each gossypol enantiomer, the oxidation was conducted quickly at room temperature and we observed by HPLC (results not shown) that the recovered gossypolone products were chemically pure (97%). Their CD curves, however, were not mirror images of each other (Fig. 2**C**) and they became completely asymmetric if their solutions were heated at 60 °C for 1 h (Fig. 2**D**). These data indicate that the gossypolone enantiomers racemize quickly at room temperature.

Since the gossypolone enantiomers could not be prepared by direct synthesis, a separation of chiral gossypolone Schiff's bases was also tried. For this, we reacted racemic gossypolone with chiral amines and attempted to separate the resulting diastereomers by chromatography on achiral stationary phases [20]. This technique has been used by Matlin and Zhou [21] to separate gossypol diastereomers, which were subsequently hydrolyzed to recover chiral gossypol.

To test this method, racemic gossypol was treated with *S*-(+)-2-aminobutane (Fig. 3, **I**) and *R*-(+)-2-amino-3-phenyl-1-propanol (Fig. 3, **II**), and the reaction mixtures were analyzed by HPLC. In both cases, the gossypol diastereoisomer Schiff's bases were separated; however, the separation with *S*-(+)-2-aminobutane was less complete at the concentration tested.

The same procedure was applied to the Schiff's bases of gossypolone formed with the same chiral amines. At room temperature, neither pair of gossypolone diastereomers was separable. For the *S*-(+)-2-aminobutane derivatives, the diastereomers had essentially the same retention time (Fig. 4, **I**). For the *R*-(+)-2-amino-3-phenyl-1-propanol derivatives, the diastereomers displayed different retention times, but rapid racemerization occurred during the chromatography resulting in a broad intermediate region between the peaks (Fig. 4, **III**). Reducing the column temperature to 0 °C resulted in good separation of the *R*-(+)-2-amino-3-phenyl-propanol-gossypolone diastereomers (Fig. 4, **IV**).

Our results indicate that gossypolone Schiff's bases are not optically stable at room temperature. Although Schiff's bases of gossypol have also been reported to racemize [22], they are more stable than Schiff's bases of gossypolone.

The results may be compared to some modeling results for gossypol and anhydrogossypol. Using molecular mechanics, Jaroszewski et al. [1] showed that anhydrogossypol was more likely to racemize than gossypol. Their calculations

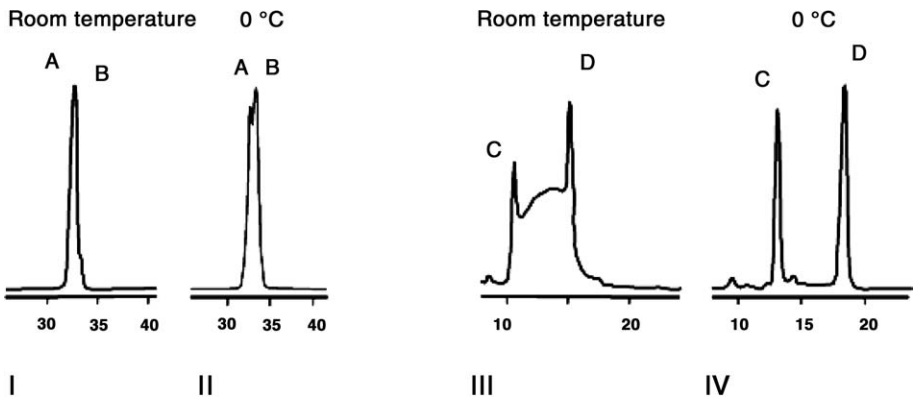

Fig. 4. HPLC separation of diastereomeric gossypolone Schiff's bases on reverse-phase C8 column: *S*-(+)-2-aminobutane gossypolone diastereomers at room temperature (**I**) and at 0 °C (**II**) and *R*-(+)-2-amino-3-phenyl-1-propanol gossypolone diastereomers at room temperature (**III**) and at 0 °C (**IV**).

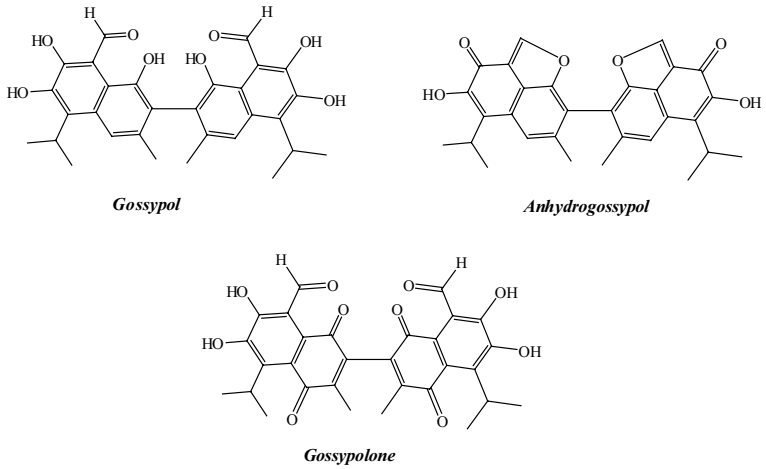

Fig. 5. Chemical structures of gossypol, gossypolone and anhydrogossypol.

suggest that the formation of bonds between the peri substituent at the C1 and C8 positions of the anhydrogossypol naphthalene rings increases the separation of the oxygen atoms away from the pivot bond, decreasing their steric interaction during rotation of the rings. For gossypolone, a similar decrease in the steric hindrance for the rotation would be expected in view of the shorter bond lengths of the carbonyl oxygen atoms in gossypolone compared with the hydroxyl bond length of gossypol (the carbonyl bond length of crystalline gossypolone is 1.22 Å [23] and the corresponding hydroxyl bond length of crystalline gossypol/acetic acid (1:1) is 1.35 Å [24]) (Fig. 5).

The racemic *S*-(+)-2-amino-butane-gossypolone-imine was also analyzed by ^{1}H NMR (600 MHz). At 300 K, the compound gives a simple spectrum (Fig. 6**A**). Reducing the temperature to 280 K resulted in a doubling of some signals, consistent with a slower interconversion of the diastereomers (Fig. 6**B**).

3. Conclusions

In a recent publication, the mechanism of gossypol-induced cell growth inhibition and cell death was investigated [25]. The action of gossypol on cancer cells is likely to be complex and involve multiple activities at various sites. These findings suggest that appropriate derivatives of gossypol could trigger apoptosis of cancer cells by targeting specific receptors.

The search for new anticancer compounds retaining the selective toxicity of gossypol without causing drug resistance remains a challenge. Nevertheless, these results show that some derivatives of enantiomeric gossypol, particularly (–)-gossypol, may represent promising leads to explore the multi-effect toxicity of gossypol.

We also observed that both gossypolone and its ethylamine derivatives do not exist as differentiable atropoisomers at room temperature. Still, gossypolone shares with gossypol high antitumor activity even for a multi-drug resistant cell line. The rapid racemization of gossypolone-based compounds may promote their association to the same receptors sites that give (–)-gossypol its enhanced antitumor effects. This suggests that gossypolone derivatives may also lead to promising new anticancer agents.

4. Experimental

4.1. Chemistry

Racemic gossypol (as gossypol / acetic acid (1:1)) was obtained as previously described [15]. Gossypol enantiomers were prepared by growing large enantiomorphic single crystals of gossypol/acetone (1:3) [16]. The chirality of each crystal was determined by HPLC separation of diastereomers formed with *R*-(–)-2-amino-1-propanol. Crystals containing the same enantiomer were combined, and the occluded acetone was removed by storing the preparations under vacuum for 4 days [16]. Reagents and solvent were purchased from Fluka Chemie AG. Chiral amines were from Acros Organics and Sigma-Aldrich.

Mass spectra were determined at the Service de Spectrometrie de Masse de I'Institut de Chimie des Substances Naturelles on AEI MS50 or Navigator-Thermoquest spectrometers. NMR spectra were recorded in $COCl_3$ on Bruker AM300 or DRX600 spectrometers. Circular dichroism was measured with a JASCO J-810 spectropolarimeter, and optical rotation was measured with a JASCO P-1010 polarimeter.

Analytical-scale HPLC work was performed with a SpectraSYSTEM P1000XR pumping system and a reverse-phase Alltima (4.6 mm i.d. × 250 mm) C8 column with isocratic elution at a flow rate of 1 ml/min. Two mobile phases were used: (I): 20% A and 80% B, (II): 30% A and 70% B. A was 95:5 (v/v) water/CH_3CN with TFA added to a final concentration of 0.1%, and B was CH_3CN also with TFA added to a final concentration of 0.1%. Compounds were detected with a Waters Corp. Model 996 photo-diode array detector operated from 254 to 400 nm and figures were recorded at 254 nm.

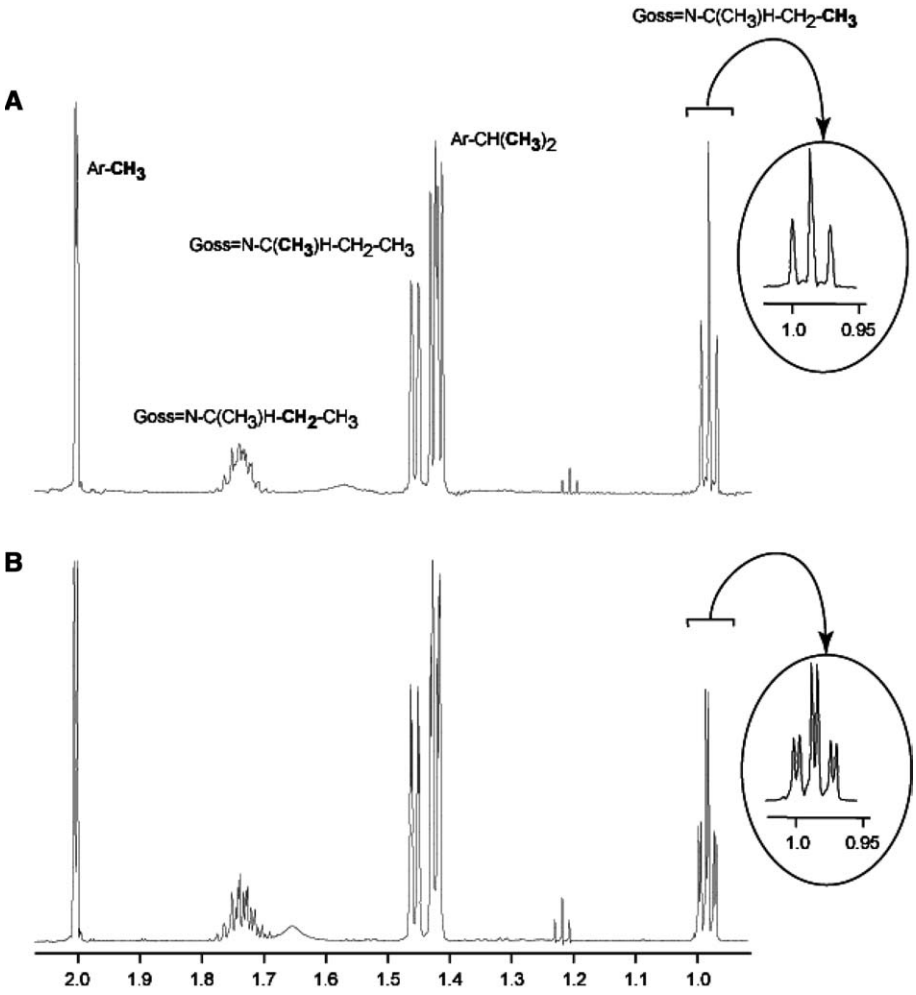

Fig. 6. ^{1}H NMR of the gossypolone Schiff's base of *S*-(+)-2-amino-butane at 300 K (**A**) and 280 K (**B**).

Synthesis of Schiff's bases were as described in our previously publication [15].

Ethylimine of (–)-*gossypol* $[\alpha]_D = -736°$ ($c = 0.03$ mg/ml; $CdCl_3$).

Ethylimine of (+)-*gossypol* $[\alpha]_D = +743°$ ($c = 0.03$ mg/ml; $CdCl_3$).

4.1.1. Gossypol Schiff's base with S-*(+)-2-aminobutane*

4.1.1.1. 8,8′-Bis[(sec-butylimino)-methyl]5,5′-diisopropyl-3,3′-dimethyl-[2,2′]binaphthalenyl-1,6,7,1′,6′,7′-hexaol. Yield 70%; HPLC: *k′* (I) = 4.64 and 4.86; ESI-MS: *m/z* 627 (M – H$^-$); ^{1}H NMR (300 MHz, $CdCl_3$): 0.96 (t, 6H, J = 7.3 Hz, 2 N–CH(CH_3)CH_2*CH_3*), 1.36 (d, 6H, J = 6.6 Hz, 2 N–CH(*CH_3*)CH_2CH_3), 1.52; 1.53 (d, 12H, J = 7.0 Hz, 2 HC–(*CH_3*)$_2$), 1.69 (m, 4H, 2 N–CH(CH_3)*CH_2*CH_3), 2.10 (s, 6H, 2 Ar-*CH_3*), 3.42 (m, 2H, 2 N–*CH*(CH_3)CH_2CH_3), 3.74 (m, 2H, J = 7.0 Hz, 2 *HC*–(CH_3)$_2$), 5.56; 7.58; 8.01(s, 6H, OH at 1,1′,6,6′,7,7′ positions), 9.64; 9.68 (s, 2H, 2 *HC*–NH), 13.45 (sl, 2H, 2 HC–*NH*).

4.1.2. Gossypol Schiff's base with D-*(+)-2-amino-3-phenyl-1-propanol*

4.1.2.1. 8,8′-Bis[(1-hydroxymethyl-2-phenylethylimino)-methyl]5,5′-diisopropyl-3,3′-dimethyl-[2,2′]binaphthalenyl-1,6,7,1′,6′,7′-hexaol. Yield 69%; HPLC: *k′* (I) = 1.51 and 2.43; ESI-MS: *m/z* 785 (MH$^+$); ^{1}H NMR (300 MHz, $CdCl_3$): 1.51; 1.53 (d, 12H, J = 7.0 Hz, 2 HC–(*CH_3*)$_2$), 2.07 (s, 6H, 2 Ar-*CH_3*), 2.96 (m, 4H, 2 N-CH(*CH_2*OH)$CH_2C_6H_5$), 3.74 (m, 8H, 2 *HC*–(CH_3)$_2$); 2 N–*CH*(CH_2OH)*CH_2*C_6H_5), 7.21 (m, 10H, 2 *C_6H_5*), 7.56; 7.97 (s, 4H, 4 *OH* at 1,1′,6,6′ positions), 9.45; 9.54 (sd, 2H, 2 *HC*–NH), 13.4 (sl, 2H, 2 HC–*NH*).

4.1.3. Gossypolone Schiff's base with S*(+)-2-aminobutane*

4.1.3.1. 8,8′-Bis-(sec-butylimino-methyl)-6,7,6′,7′-tetrahydroxy-5,5′-diisopropyl-3,3′-dimethyl-[2,2′]binaphthalenyl-1,4,1′,4′-tetraone. Yield 73%; *k′* (I) = 3.44; ESI-MS: *m/z* 627 (M – H$^-$); ^{1}H NMR (300 MHz, $CdCl_3$): 0.97 (t, 6H,

J = 7.3 Hz, 2 N-CH(CH_3)CH_2*CH_3*), 1.39; 1.41 (d, 12H, J = 7.0 Hz, 2 HC–(*CH_3*)$_2$), 1.45 (d, 6H, J = 6.6 Hz, 2 N–CH(*CH_3*)CH_2CH_3), 1.71 (m, 4H, 2 N–CH(CH_3)*CH_2*CH_3), 1.98 (s, 6H, 2 Ar-*CH_3*), 3.45 (m, 2H, 2 N-*CH*(CH_3)CH_2CH_3), 3.84 (m, 2H, J = 7.0 Hz, 2 *HC*–(CH_3)$_2$), 7.16; 8.08 (s, 4H, *OH* at 6,6′,7,7′ positions), 9.64; 9.59 (sl, 2H, 2 *HC*–NH), 14.87 (sl, 2H, 2 HC–*NH*).

4.1.4. Gossypolone Schiff's base with D-(+)-2-amino-3-phenyl-1-propanol

4.1.4.1. 6,7,6′,7′-Tetrahydroxy-8,8′-bis[(1-hydroxymethyl-2-phenylethylimino)-methyl]-5,5′-diisopropyl-3,3′-dimethyl-[2,2′]binaphthalenyl-1,4,1′,4′-tetraone. Yield 64%; *k′* (I) = 1.22 and 1.84; ESI-MS: *m/z* 811 (M – H^-); ^{1}H NMR (300 MHz, $CdCl_3$): 1.43 (dd, 12H, J = 7.0 Hz, 2 HC–(*CH_3*)$_2$), 1.95 (s, 6H, 2 Ar-*CH_3*), 2.97 (m, 4H, 2 N–CH(*CH_2*OH) $CH_2C_6H_5$), 3.83 (m, 8H, 2 *HC*–(CH_3)$_2$), 2 N–*CH*(CH_2OH) *CH_2*C_6H_5), 7.24 (m, 14H, 2 *C_6H_5, OH* at 6,6′,7,7′ positions).

4.2. Biological studies—cytotoxity

As previously described [15] IC_{50} values are the mean of three independent experiments, each performed at least in duplicate.

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