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Antifungal activity of alkyl and heterocyclic aza-derivatives of gossypol as well as their complexes with $NaClO_4$ against *Fusarium oxysporum* f. sp. *lupini*

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ABSTRACT

Eight alkyl and six heterocyclic aza-derivatives of gossypol (**2**–**15**) have been synthesized using gossypol (**1**) extracted from *Gossypium Herbaceum* cottonseeds. The ability of gossypol aza-derivatives to form complexes with $NaClO_4$ has been investigated by electrospray ionisation (ESI) mass spectra recorded in the positive and negative ion detection modes. The gossypol aza-derivatives have been characterized by FT-IR, ^1H and ^{13}C NMR spectroscopic methods and subsequently tested for their antifungal properties against *Fusarium oxysporum*. Four alkyl aza-derivatives (**2**–**5**), present in the enamine–enamine tautomeric form, have shown activity comparable or higher than that of gossypol against this fungus. To improve the antifungal activity the complexes of the most active compounds **2**–**5** with $NaClO_4$ were prepared. Complexes of **2** and **5** with $NaClO_4$ have shown antifungal activity higher than that of the uncomplexed compounds.

Gossypol is a yellow pigment, present in various parts of the cotton plants[1], which has drawn the attention of many scientist because of its wide biological activities such as contraceptive,[2] anticancer,[3] antiviral[4] or antimicrobial.[5] Despite these interesting and potentially useful effects, the application of gossypol as a therapeutic or antimicrobial agent still remains a challenge because of a number of serious sides effects.[6] A convenient way to obtain less toxic compounds based on gossypol is to block the gossypol aldehyde groups by their conversion into, for example, Schiff bases, hydrazones, nitriles etc. Up to now various thio-, aza- and aza-modifications of gossypol have been tested in order to obtain less toxic derivatives which would extend the fields of its application.[7] From earlier studies it is known that the tautomeric forms of gossypol aza-derivatives (Fig. 1) depend on the nature of the substituent directly bonded to N16 and N16′ atoms.[8]

The fungus *Fusarium oxysporum* is a worldwide serious problem because induction of wilt disease in a wide range of host plants such as, for example, cereals, tomatoes, potatoes, bananas and watermelons.[9] This plant pathogenic fungus strain produces poisonous chemical compounds like mycotoxins (e.g., Fusaric acid) which contaminate harvested crops. Ingestion of cereals vegetables or fruits affected by the disease evoked by the *Fusarium oxysporum* may give rise to allergic symptoms or be carcinogenic by long-term consumption in humans and animals due to the presence of mycotoxins produced by the fungus.[10]

Gossypol antifungal activity is concerned with its protecting role of cotton plants against many pathogens like, for example, *Rhizoctonia Solani*.[11] Recently, Turco et al. investigated in vitro the effect of gossypol and its mixture with NaCl on conidial germination and viability of *Fusarium oxysporum* sp. Vasinfectum isolates.[12] These authors have reported that the inhibitory effect against *Fusarium oxysporum* is significant at gossypol concentrations between 10 and 20 mg/L, while the addition of the salt reduces the antifungal activity of gossypol. Up to now many derivatives, especially fluorine derivatives of various heterocycles[13], have been tested against *Fusarium oxysporum* in contrast to gossypol aza-derivatives whose antifungal activity in this field is unexplored. This fact has motivated our group to undertake synthesis and biological tests of a series of gossypol aza-derivatives containing alkyl and heterocyclic moieties and their complexes with $NaClO_4$ against the pathogenic fungus. Gossypol was extracted[14] from cottonseeds of *Gossypium herbaceum* and subsequently converted into its eight Schiff bases and six hydrazones by a simple condensation of amines and hydrazines with the two aldehyde groups of gossypol in dichloromethane.[15]

The structures of all Schiff bases and hydrazones of gossypol were determined by the ESI MS, FT-IR and NMR methods.[16,17] The FT-IR spectra of gossypol Schiff bases and gossypol hydrazones are significantly different in the ν(C=O) range. In the spectra of gossypol Schiff bases (**2**–**9**) an intense band at about 1640 cm^{-1}, assigned to the

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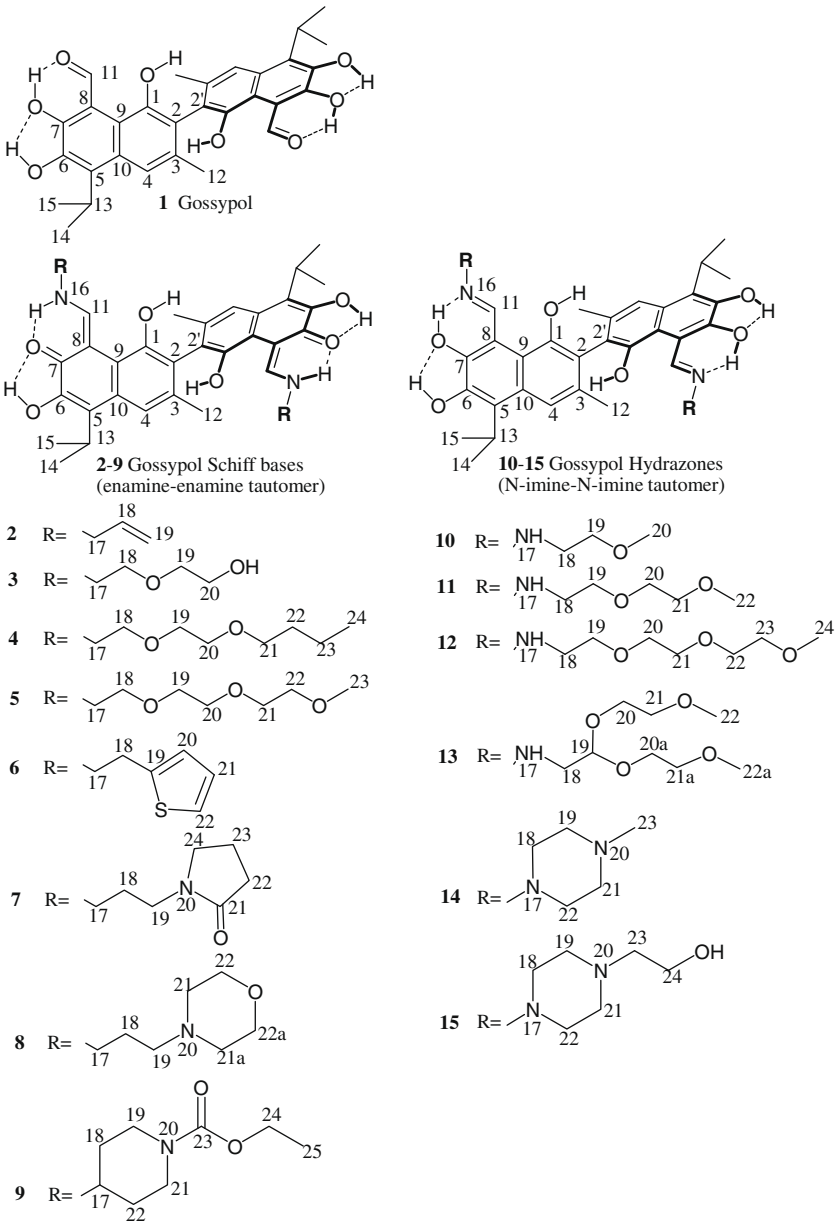

Figure 1. Chemical structure together with the atom numbering of gossypol and its aza-derivatives studied.

ν[C(7)=O] stretching vibrations of carbonyl group is observed. Instead of this band in the FT-IR spectra of gossypol hydrazones (**10**–**15**) a medium intense band at about 1610 cm^{-1}, assigned to the overlapped ν(C=N) and ν(C=C) stretching vibrations, is present. These spectral features already indicate the presence of the enamine–enamine and *N*-imine–*N*-imine tautomeric forms in gossypol Schiff bases and gossypol hydrazones, respectively.

Valuable information on these tautomeric forms can be also obtained from the chemical shifts of the C(7) carbon atom signals in the ^{13}C NMR spectra. In the spectrum of **1** the signal of C(7) carbon atom was found at 151.4 ppm in CD_3CN[18], whereas the signal of C(7) carbon atom in the spectra of **10**–**15** in DMSO-d_6 was in the range 150.4–151.6 ppm. The chemical shift of the C(7) signals of **1**, similarly to those of **10**–**15**, are in the range characteristic of the C–OH carbon atoms of phenols demonstrating the presence of aldehyde–aldehyde and *N*-imine–*N*-imine tautomeric forms within **1** and **10**–**15**, respectively. The C(7) resonances of **2**–**9** are positioned in different range 171.8–172.7 ppm demonstrating the double bond character of C(7)=O bond in naphthalene ring. The strongest engagement of the N(16)-H and O(7)-H protons in the N(16)-H···O(7) and N(16)···H-O(7) intramolecular hydrogen bonds is well reflected in their ^1H chemical resonances in DMSO-d_6, that is, δ_{H16} = 13.17–13.21 ppm /**2**–**9**/ and δ_{H7} 14.15–14.54 ppm /**10**–**15**/, respectively. Relatively weakly hydrogen bonded are the protons of O(1)H groups because their chemical shifts in DMSO-d_6, irrespectively of the type of tautomer, are found in a similar narrow range δ_{H1} = 7.69–7.97 ppm. The presence of different tautomers within gossypol Schiff bases and hydrazones is also reflected in the chemical shifts of O(6)-H protons which are hydrogen-bonded to the oxygen atoms of C(7)=O carbonyl (δ_{H6} = 8.41–8.96 ppm for **2**–**9**) and C(7)-OH phenol groups (δ_{H6} = 7.99–8.13 ppm for **10**–**15**), respectively. A comparison of the position of C(7) carbon atom signals in the ^{13}C NMR spectra of gossypol Schiff base (**4**)[16] and gossypol hydrazone (**14**)[17], recorded in DMSO-d_6, with those taken in $CDCl_3$[8] indicates the preservation of the respective tautomeric forms in different solvents.

This result confirms that gossypol Schiff bases (**2**–**9**) and gossypol hydrazones, irrespectively of the type of substituent and kind

of solvent, exist in different tautomeric forms in solutions, stabilized by several intramolecular hydrogen bonds (Fig. 1).

Fourteen gossypol aza-derivatives dissolved in DMSO (**2**–**15**), were preliminary examined for their antifungal activity against *Fusarium oxysporum* at concentrations of 20 μg/mL and 1 μg/mL following the method described in Ref. 19. For comparison **1**, DMSO, $NaClO_4$/DMSO as well as thiophanate-methyl as a standard antimicrobial drug were also tested (Table 1). A comparison of the antifungal test results, collected in Table 1, demonstrates that at the 20 μg/mL and 1 μg/mL concentrations gossypol Schiff bases (**2**–**5**), present in the enamine–enamine tautomeric forms, show antifungal activity in contrast to gossypol hydrazones (**10**–**15**),

Table 1
Antifungal activity of gossypol (**1**) and its alkyl and heterocyclic aza-derivatives (**2**–**15**) as well as aza-derivative (**2**–**5**) complexes with $NaClO_4$, radial growth inhibition of fungi (Ā) (mm)

Compound	Concn (μg/mL)	Ā after 48 h (mm)	Ā after 96 h (mm)	Ā after 144 h (mm)	Ā after 192 h (mm)	Improvement of fungi growth inhibition relative to DMSO (%) after 192 h
1	20	14.88 ± 0.79	25.25 ± 0.88	35.00 ± 0.66	46.00 ± 0.66	22.4
	1	17.25 ± 0.57	26.25 ± 0.88	36.75 ± 0.88	47.25 ± 1.10	20.3
	0.5	18.63 ± 0.92	27.88 ± 0.79	39.50 ± 0.94	50.50 ± 0.94	14.8
	0.1	19.63 ± 0.92	30.63 ± 0.92	42.75 ± 0.88	55.00 ± 0.94	7.2
2	20	14.63 ± 0.64	24.75 ± 0.57	34.63 ± 0.64	45.88 ± 0.79	22.6
	1	17.13 ± 0.79	26.00 ± 0.94	36.63 ± 0.92	47.00 ± 0.94	20.7
	0.5	18.13 ± 0.79	27.25 ± 0.88	38.88 ± 0.79	50.00 ± 0.94	15.6
	0.1	19.25 ± 0.88	29.88 ± 1.03	41.63 ± 0.92	53.75 ± 0.88	9.3
3	20	14.38 ± 0.64	24.50 ± 0.94	34.50 ± 0.94	45.25 ± 0.88	23.6
	1	16.00 ± 0.66	25.75 ± 0.88	35.88 ± 1.23	46.25 ± 1.10	21.9
	0.5	17.63 ± 0.64	26.50 ± 0.66	38.00 ± 1.32	49.50 ± 0.94	16.5
	0.1	18.13 ± 1.03	28.50 ± 1.15	40.38 ± 0.92	52.63 ± 0.92	11.2
4	20	13.50 ± 0.66	23.63 ± 0.64	33.25 ± 0.88	43.63 ± 0.92	26.4
	1	14.25 ± 0.57	23.50 ± 0.94	33.88 ± 1.23	44.00 ± 0.94	25.7
	0.5	15.13 ± 0.44	24.63 ± 0.64	34.75 ± 1.28	45.13 ± 1.03	23.8
	0.1	16.38 ± 1.13	25.63 ± 1.61	36.75 ± 1.10	49.25 ± 1.44	16.9
5	20	14.13 ± 0.79	24.13 ± 0.79	34.13 ± 1.39	44.00 ± 1.15	25.7
	1	14.88 ± 1.03	25.00 ± 0.66	35.25 ± 0.57	45.75 ± 0.88	22.8
	0.5	16.38 ± 1.74	25.75 ± 1.59	36.88 ± 1.68	48.50 ± 1.48	18.1
	0.1	16.75 ± 1.10	27.00 ± 0.94	39.25 ± 1.44	51.75 ± 1.28	12.7
6	20	19.38 ± 0.64	31.75 ± 0.57	45.75 ± 0.57	58.75 ± 0.57	0.8
	1	19.63 ± 0.64	32.00 ± 0.66	46.00 ± 0.66	59.13 ± 1.03	0.2
7	20	19.50 ± 0.66	31.88 ± 0.44	45.88 ± 0.44	58.88 ± 0.44	0.6
	1	19.75 ± 0.57	32.13 ± 0.44	46.13 ± 0.79	59.25 ± 1.10	0.0
8	20	19.25 ± 0.88	31.63 ± 0.64	45.63 ± 0.64	58.63 ± 0.64	1.0
	1	19.50 ± 0.94	31.88 ± 0.79	45.88 ± 0.79	59.00 ± 1.32	0.4
9	20	19.63 ± 0.64	32.13 ± 0.44	46.00 ± 0.66	59.00 ± 0.66	0.4
	1	19.88 ± 0.44	32.25 ± 0.57	46.25 ± 0.88	59.25 ± 1.10	0.0
10	20	19.50 ± 0.66	31.88 ± 0.79	45.88 ± 1.03	58.88 ± 1.03	0.6
	1	19.75 ± 0.57	32.13 ± 0.79	46.13 ± 1.23	59.00 ± 1.15	0.4
11	20	19.38 ± 0.64	31.75 ± 1.10	45.88 ± 1.23	58.63 ± 1.61	1.0
	1	19.63 ± 0.64	32.00 ± 1.15	45.88 ± 0.79	58.88 ± 0.79	0.6
12	20	19.88 ± 1.03	32.00 ± 1.48	46.13 ± 1.03	58.88 ± 1.39	0.6
	1	19.88 ± 0.44	32.25 ± 1.10	46.13 ± 0.79	59.13 ± 1.03	0.2
13	20	19.63 ± 0.92	31.63 ± 1.31	45.50 ± 0.66	58.38 ± 1.47	1.5
	1	19.75 ± 0.88	32.13 ± 1.03	45.63 ± 0.92	58.63 ± 1.13	1.0
14	20	19.25 ± 0.57	31.63 ± 0.92	45.75 ± 0.88	58.50 ± 1.15	1.3
	1	19.38 ± 0.64	31.88 ± 1.23	45.75 ± 0.57	58.75 ± 0.57	0.8
15	20	19.50 ± 0.94	31.50 ± 1.15	45.38 ± 0.64	58.25 ± 1.28	1.7
	1	19.63 ± 0.64	32.00 ± 0.94	45.50 ± 0.66	58.50 ± 0.94	1.3
2+$NaClO_4$	1	15.63 ± 0.64	24.13 ± 0.79	34.88 ± 0.79	45.25 ± 0.88	23.6 (3.7)**
	0.5	17.13 ± 0,79	26.25 ± 1.10	37.00 ± 1.32	47.38 ± 1.13	20.0 (5.2)**
	0.1	18.50 ± 0.66	28.75 ± 0.88	39.63 ± 0.64	51.13 ± 1.03	13.7 (4.9)**
3+$NaClO_4$	1	15.50 ± 0.94	24.75 ± 1.28	34.75 ± 0.88	45.38 ± 1.31	23.4 (1,9)**
	0.5	17.25 ± 0.57	26.25 ± 0.57	37.50 ± 0.66	49.00 ± 0.94	17.3 (1.0)**
	0.1	17.63 ± 1.13	28.00 ± 1.15	39.75 ± 1.10	52.00 ± 1.15	12.2 (1.2)**
4+$NaClO_4$	1	14.00 ± 0.94	23.13 ± 1.03	33.50 ± 0.94	43.63 ± 1.13	26.4 (0.8)**
	0.5	14.88 ± 0.79	24.38 ± 0.64	34.50 ± 1.15	44.88 ± 1.23	24.3 (0.8)**
	0.1	16.13 ± 0.79	25.38 ± 1.13	36.50 ± 0.66	48.88 ± 1.39	17.6 (0.8)**
5+$NaClO_4$	1	14.50 ± 0.94	23.88 ± 0.44	33.88 ± 1.80	44.00 ± 1.62	25.7 (3.8)**
	0.5	15.00 ± 1.15	24.00 ± 0.94	34.50 ± 0.94	45.00 ± 0.94	24.1 (7.2)**
	0.1	15.50 ± 1.62	24.75 ± 1.84	35.50 ± 1.75	46.13 ± 1.54	22.1 (10.9)**
DMSO	—	19.88 ± 0.79	32.38 ± 0.92	46.38 ± 1.13	59.25 ± 1.44	—
$NaClO_4$/DMSO	1*	19.75 ± 1.10	31.75 ± 1.72	45.88 ± 1.23	58.75 ± 1.10	0.8
	0.5*	19.75 ± 0.57	32.13 ± 1.23	46.00 ± 0.66	58.88 ± 1.23	0.6
	0.1*	19.88 ± 0.44	32.38 ± 0.92	46.00 ± 0.66	59.25 ± 0.88	0.0
Thiophanate-methyl	20	17.00 ± 0.94	30.63 ± 0.64	41.50 ± 0.94	53.13 ± 1.03	10.3
	1	19.00 ± 0.94	31.88 ± 1.03	42.75 ± 0.88	54.00 ± 1.15	8.9
	0.5	20.13 ± 1.03	32.88 ± 1.23	44.75 ± 1.10	55.38 ± 0.92	6.5
	0.1	21.38 ± 0.92	33.50 ± 0.94	46.13 ± 0.79	57.13 ± 0.79	3.6
Control	—	23.00 ± 0.94	38.63 ± 1.31	57.38 ± 1.31	70.25 ± 1.28	—

The error for the measurements was determined for the $\alpha = 0.01$.
t-Student statistical test was performed.
* Amount of $NaClO_4$ used as in the tests of the gossypol derivative-$NaClO_4$ complexes at respective concentrations.
** Improvement of fungi growth inhibition after complexation relative to respective gossypol aza-derivative [%] after 192 h

present in the *N*-imine–*N*-imine tautomeric forms (Supplementary data—Scheme 1a). Despite the existence of the enamine–enamine tautomeric form within all Schiff bases, only some of them have very promising antifungal activity, that is, gossypol Schiff bases **2**–**5** are active in contrast to the gossypol Schiff bases **6**–**9** which show no activity (Table 1).

Thus, the antifungal activity of compounds **2**–**5** is a result of both, the presence of the enamine–enamine tautomeric form as well as alkyl chains within the gossypol aza-derivative structures. Alkyl chains of **2**–**5** probably enable the interaction with the cell walls of *Fusarium oxysporum*, built of chitin and β-1,3-glucan, responsible for the rigidity of the cell wall of the fungus.[20]

Further biological tests of the active compounds **2**–**5**, performed at even lower concentrations (0.5 μg/mL and 0.1 μg/mL), still revealed significant activity of these derivatives.

Earlier studies have shown that gossypol aza-derivatives, in contrast to **1**, are able to form stable complexes with Na^+ cations and ClO_4^- anion[21] (Supplementary data—Scheme 2). The next step of our investigations was to check if the addition of the salt at lower concentrations (1 μg/mL, 0.5 μg/mL; 0.1 μg/mL) influences the activity of the compounds being both the best ligands toward $NaClO_4$ (Supplementary data—Scheme 3a and b) and the most active ones (**2**–**5**).

The results of biological tests of the equimolar mixtures **2**–**5** with $NaClO_4$ are shown in Table 1. Generally, these data demonstrate that the addition of the salt improves the activity of all gossypol Schiff bases **2**–**5** containing alkyl substituents (Supplementary data—Scheme 1b), while the greatest improvement in the antifungal activity was noted for compounds **2** and **5**. To explain the dependence of the activity improvement of **2**–**5** after addition of $NaClO_4$, respective electrospray ionization mass spectra of 4:1:1:1:1 mixtures of $NaClO_4$ and **2**–**5** compounds in the negative (ESI^-) and positive (ESI^+) ion detection modes were recorded (Supplementary data—Scheme 4a and b). The ESI^+ and ESI^- mass spectra reveal that the ClO_4^- anion is the best complexed by **2** while the Na^+ cation is the best complexed by compound **5** because the $[\mathbf{2}+ClO_4]^-$ and $[\mathbf{5}+Na]^+$ signals are of the highest intensities. The signals of $[\mathbf{3}+ClO_4]^-$, $[\mathbf{3}+Na]^+$, $[\mathbf{4}+ClO_4]^-$, $[\mathbf{4}+Na]^+$ ions in the mass spectra are of medium and low abundance indicating moderate affinity of compounds **3** and **4** both toward the Na^+ cation and ClO_4^- anion. Taking this result into account, we can conclude that the greatest improvement in the antifungal activity of compounds **2** and **5** after addition of the salt can be related to good complexation abilities of these ions. As we have shown earlier, the complexation process of ClO_4^- anion and Na^+ cation by the gossypol aza-derivatives, which contain alkyl moieties, evoked the conformational change and in consequence the exposition of the hydrophobic part of the molecules to the outer environment[21], for example, to the lipid bilayers of *Fusarium oxysporum*.

In the present work, we have synthesized fourteen gossypol aza-derivatives (**2**–**15**) and their structures have been investigated by spectroscopic methods. Among all compounds studied, only **2**–**5** which contain alkyl moieties and are present as the enamine–enamine tautomers have shown promising activity against plant pathogen fungus—*Fusarium oxysporum* at various concentrations. Furthermore, we have demonstrated that the addition of $NaClO_4$ salt to the active compounds **2** and **5**, showing the best complexation properties toward the anion or the cation, improves their antifungal properties.

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Supplementary data

Exemplary biological test results as well as the ESI^- and ESI^+ mass spectrometric data. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.02.051.

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14. Extraction of gossypol: Cottonseeds of *Gossypium herbaceum* (200 g) were extracted first with 2 L of petroleum ether (40–60° C) for 48 h and next with 2 l of diethyl ether for 36 h. The crude extract was filtered off and subsequently evaporated under reduced pressure to 300 mL and treated by the 5 mL of the glacial acetic acid. After 48 h the gossypol-acetic acid complex precipitated and was filtered off (0.839 g). The crude gossypol-acetic acid complex was dissolved in the 1:1 mixture of H_2O–diethyl ether (300 mL) and stirred vigorously for 36 h. After this time the solvents were evaporated to dryness under reduced pressure (0.720 g; Yield: 95.7%). Crude gossypol was recrystallised from the ethanol solution giving 0.660 g of a yellow solid. ESI^+ MS $[M+H]^+$ m/z = 519.
15. General procedure for the synthesis of gossypol Schiff bases (**2**–**5**) and gossypol hydrazones (**6**–**9**): To a solution of (**1**) (500 mg, 0.96 mmol) in dichloromethane (30 mL) the respective amines or hydrazines in dichloromethane (40 mL) were added (1.92 mmol). The mixture was first stirred at 35° C for 5 h and then solvent was evaporated to the 40 mL of total volume. Next the mixture was cooled to the room temperature and subsequently the 10 mL of methanol was added. The products were precipitated, filtered off and dried under reduced pressure. Crude precipitates were recrystallised from 1:1 dichloromethane-ethanol mixture. Gossypol aza-derivatives were obtained as orange or orange–brown solids (Yield from 82% to 91%).
16. *Selected spectral data for* (**4**): ESI-MS (m/z): 805 $[M+H]^+$; ^1H NMR (δ ppm in DMSO-d_6): 7.78 (2H, s, OH-1), 7.45 (2H, s, H-4), 8.41 (2H, s, OH-6), 9.75 (2H, d, J = 12.9 Hz, H-11), 1.94 (6H, s, H-12), 3.69 (2H, sept, J = 7.2 Hz, H-13), 1.43 and 1.45 (d, J = 7.2 Hz, H-14 and H-15), 13.21 (2H, m, J = 12.9 Hz and J = 6.8 Hz, NH-16), 3.65 (4H, m, J = 6.8 Hz, J = 4.1 Hz, H-17), 3.55 (4H, m, J = 4.1 Hz and J = 8.7 Hz, H-18), 3.47 (2H, t, J = 4.1 Hz, H-19), 3.64 (4H, m, J = 4.1 Hz, H-20), 3.32 (4H, dt*, J = 6.5 Hz and J = 6.6 Hz, H-21), 1.37 (4H, m, J = 6.6 Hz and

J = 14.3 Hz, H-22), 1.21 (4H, m, J = 7.1 Hz, J = 7.3 Hz, J = 14.4 Hz, H-23), 0.79 (6H, dt*, J = 7.1 Hz and J = 7.3 Hz, H-24); ^{13}C NMR (δ ppm in DMSO-d_6): 149.6 (C-1), 120.0 (C-2), 131.1 (C-3), 116.5 (C-4), 126.3 (C-5), 146.3 (C-6), 171.8 (C-7), 103.1 (C-8), 115.8 (C-9), 126.9 (C-10), 162.9 (C-11), 20.1 (C-12), 26.5 (C-13), 20.3 (C-14 and C-15), 49.9 (C-17), 69.8 (C-18), 69.4** (C-19 and C-20), 70.0 (C-21); 31.3 (C-22); 18.7 (C-23) 13.7 (C-24); ^{1}H and ^{13}C resonance assignments were confirmed by the COSY, HSQC, HMBC and NOESY 2D correlations, *—double triplet, **—separated signals; FT-IR (KBr pellet): 1639 cm^{-1} ν(C=O), 1618 cm^{-1} ν(C_{arom}=C_{arom}); Elemental Anal. Calcd for $C_{46}H_{64}N_2O_{10}$: C, 68.63; H, 8.01, N, 3.48. Found: C, 68.60; H, 8.00, N, 3.45. Other gossypol Schiff bases (**2**, **3**, **5**–**9**) were characterized by us in Refs. 8a,e–g,18,21a.

17. *Selected spectral data for* (**12**): ESI-MS (m/z): 839 [M+H]$^+$; ^{1}H NMR (δ ppm in DMSO-d_6): 7.97 (2H, s, OH-1), 7.64 (2H, s, H-4), 8.13 (2H, s, OH-6), 14.59 (2H, s, OH-7), 10.04 (2H, s, H-11), 1.98 (6H, s, H-12), 3.75 (2H, sept, J = 7.5 Hz, H-13), 1.44 and 1.46 (12H, d, J = 7.5 Hz, H-14 and H-15), 6.56 (2H, s, H-17), 3.38 (4H, t, J = 7.1 Hz, H-18), 3.69 (4H, t, J = 7.1 Hz, H-19), 3.49 (4H, t, J = 5.3 Hz, H-20), ~3.56 (4H, t, J = 5.3 Hz, H-21), 3.54 (4H, t, J = 4.9 Hz, H-22), 3.49 (4H, t, J = 4.9 Hz, H-23), 3.22 (6H, s, H-24); ^{13}C NMR (δ ppm in DMSO-d_6): 149.8 (C-1), 115.6 (C-2), 132.0 (C-3), 117.4 (C-4), 126.0 (C-5), 143.8 (C-6), 150.7 (C-7), 106.9 (C-8), 114.6 (C-9), 129.7 (C-10), 151.2 (C-11), 20.0 (C-12), 26.7 (C-13), 20.2 (C-14 and C-15), 50.9 (C-18), 68.4 (C-19), 69.1 (C-20), 69.5 (C-21), 69.1 (C-22); 72.1 (C-23); 59.6 (C-24); ^{1}H and ^{13}C resonance assignments were confirmed by the COSY, HSQC, HMBC and NOESY 2D correlations; FT-IR (KBr pellet): 1608 cm^{-1} ν(C=N) + ν(C_{arom}=C_{arom}); Elemental Anal. Calcd for $C_{44}H_{62}N_4O_{12}$: C, 62.99; H, 7.45; N, 6.68. Found: C, 62.96; H, 7.41; N, 6.65. Other gossypol hydrazones (**10**, **11**, **13**–**15**) were characterized by us in Refs. 8a,h.
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19. Stock cultures of *Fusarium oxysporum* f. sp. *Lupini* were grown on PDA medium (pH 6.5) at 25 °C for 14 days. Appropriate solutions in DMSO of thiophanate-methyl, **1**, **2**–**15** as well as **2**+$NaClO_4$, **3**+$NaClO_4$, **4**+$NaClO_4$, **5**+$NaClO_4$ mixtures were added to melted PDA, so that the final concentration were respectively 20, 1, 0.5, 0.1 μg/mL. Agar discs with actively growing mycelium were cut out with sterile cork borer and placed at the center of Petri dishes. Four replicate plates were inoculated for each concentration of **1**, **2**–**15** (20 μg/mL and 1 μg/mL). For comparison four replicate plates of thiophanate-methyl (20, 1, 0.5, 0.1 μg/mL), $NaClO_4$/DMSO (1, 0.5, 0.1 μg/mL), DMSO and PDA medium (Control) were also inoculated. The plates were incubated at 25 °C and after appropriate time intervals (48 h) two diagonal measurements of radial growth inhibition of fungi for four replicate plates (eight measurements) were made. The data obtained are given in Table 1 while exemplary results of the antifungal tests performed on the active aza-derivatives and their complexes (after 192 h) are given in Scheme 1a and b (Supplementary data), respectively. The most active gossypol aza-derivatives (**2**–**5**) were further tested at lower concentrations (0.5 and 0.1 μg/mL) as well as after addition of $NaClO_4$ (gossypol aza-derivative:$NaClO_4$ equal 1:1 molar ratio) for concentrations = 1, 0.5, 0.1 μg/mL. Four replicate plates were inoculated for each sample concentration and subsequently radial growth inhibition of fungi was measured two times. The data obtained were analyzed by a t-Student test using *Statistica 8.0* (1984–2008 StatSoft. Inc., Tulsa, OK, USA) at significance level of α = 0.01.
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