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Synthesis and antimicrobial activities of some novel thiophene containing azo compounds

Abstract: A series of new thienylazothiophenes were prepared by coupling reactions of diazotized thienocoumarins with their corresponding free amines. The structures of the products **3–7** were assigned on the basis of their elemental and spectroscopic data. The HMBC and HSQC techniques were used in some cases to ascertain the structural assignments. The antimicrobial screenings of compounds **3–7** along with substituted 2-aminothiophenes **1a,b** were performed against three bacteria and three fungal species and their activities compared to those of nystatin, gentamycin and ciprofloxacin used as reference drugs. It was found that the antimicrobial activities of the tested new hybrid compounds were in some cases equal or better than those of the reference drugs.

Keywords: 2-aminothiophenes; antifungal agents; azo compounds; bathochromic effects; electrophilic substitution.

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Introduction

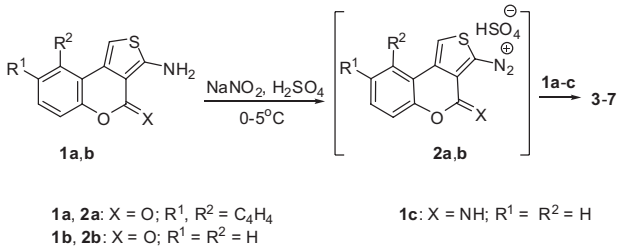
Coumarins, thiophenes and azo dyes are three classes of organic compounds that are gaining growing interest because of their various important applications in industry [1–4] and medicinal chemistry [5–9]. In continuation of our ongoing investigations [10] on the coupling reactions of substituted 2-aminothiophenes, it was thought that the combination of azo, coumarin and thiophene fragments in the same molecular framework might result in generating compounds with improved industrial and pharmaceutical applications.

The three derivatives of 2-aminothiophene **1a–c** used as starting materials in this work were prepared by applying the third version of the Gewald technique [11, 12], as previously described. The interesting results that we recently found from the antifungal activities and the effects on *Microsporum gypseum* protein profile [13] of these compounds stimulated us to carry out similar investigations on azo compounds derived from them. In the present work, we report on the synthesis and the structural elucidation of five new thienylazothiophene derivatives **3–7** obtained from substrates **1**. Along with the stating materials **1a** and **1b**, we also carried out studies on the antimicrobial activities of the new compounds against six pathogen bacterial and fungal species. The structure-activity relationships are discussed.

Results and discussion

The starting compounds **1** were synthesized by applying the two-step procedure of the Gewald methodology [14, 15]. Compounds **1** were diazotized at low temperature (0–5°C) using nitrosyl sulfuric acid and coupled with the free amines **1a–c** to afford the diazo compounds **3–7** (Scheme 1). As previously mentioned [10], when subjected to diazotization, compound **1c** first undergoes an oxidation of its imino function into the oxo moiety and the intermediate **2b** is generated.

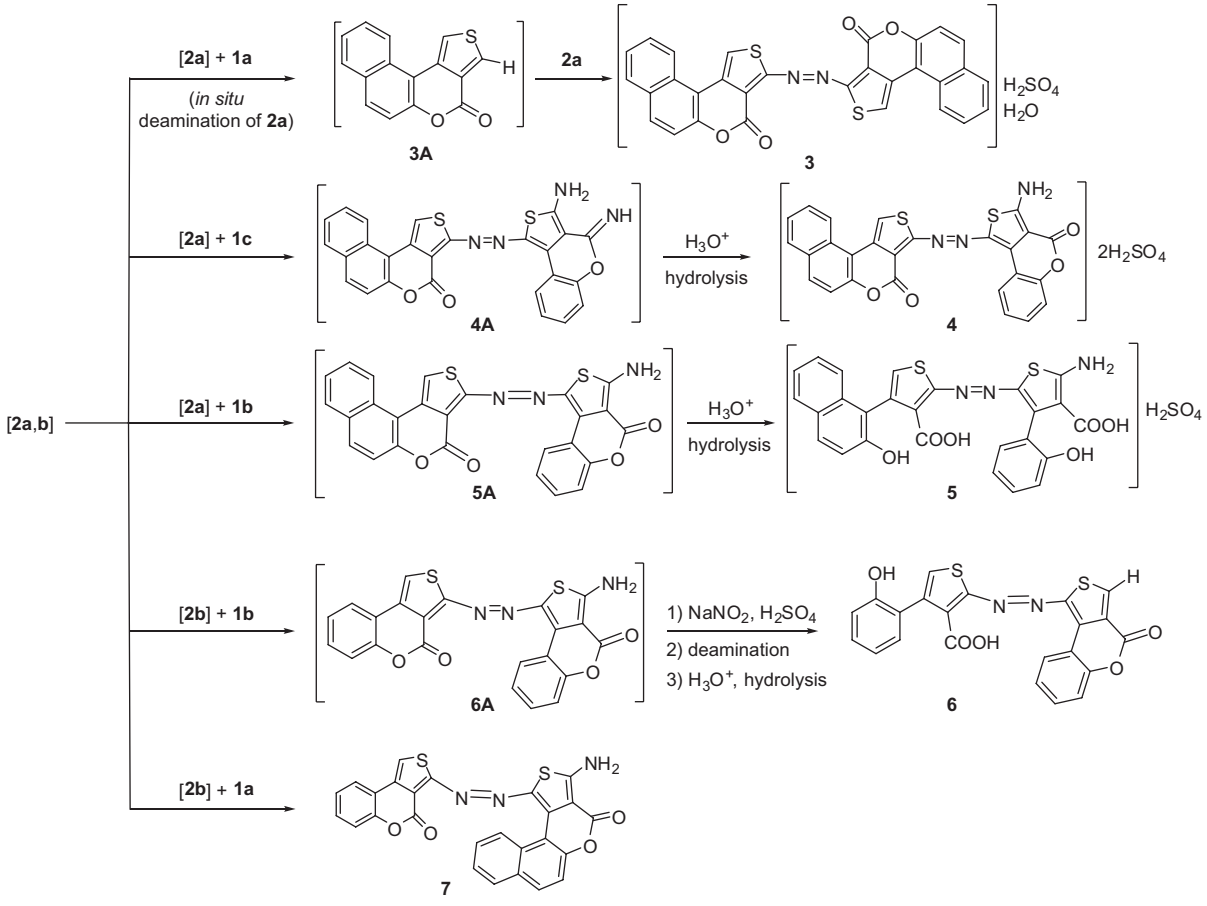
The thienyldiazonium sulfate **2a** was directly coupled *in situ* with the 2-aminothienocoumarins **1a** and **1c** to afford compounds **3** and **4**, respectively (Scheme 2). In the process of mixing compound **1a** with its diazonium salt solution (containing **2a**), it further undergoes diazotization by the nitrosyl sulfuric acid used in excess. By contrast, the *in situ* deaminated [16, 17] intermediate



Scheme 1

product **3A** most probably undergoes coupling with the thienyldiazonium sulfate **2a** to form compound **3** as the only isolated product. In the reaction of **2a** and **1c**, the normally anticipated product **4A** subsequently undergoes acid hydrolysis to give the isolated product **4**. From the reaction of **2b** and **1a** however, the coupling product **7** was isolated as expected. Under similar reaction conditions, the treatment of thienyldiazonium salt **2a** with compound **1b** gave the hydrolysis product **5** via the normally expected intermediate **5A**, as the result of the opening of the two coumarin rings. The coupling of **2b** with **1b** gave compound **6** as the only isolated product. The formation of **6** probably results from the subsequent diazotization, deamination [16, 17] and hydrolysis of one of the two coumarin rings of the primarily formed intermediate **6A** (Scheme 2).

Compound **3** was obtained in 45% overall yield in the form of red powder crystallizing with 1 molecule of H_2SO_4 and 1 molecule of H_2O. Structure **3** was assigned on the basis of the combustion analysis data which established the formula to be $C_{30}H_{18}N_2O_9S_3$. In the IR spectrum, the characteristic N-H stretching frequencies of the NH_2 group could not be seen, as expected. In addition, the appearance of the N=N stretching frequencies in the range 1440–1412 cm^{-1} confirmed the presence of the azo bridge. The ^{13}C NMR spectrum contains 15 relevant signals as expected for the symmetrical structure of **3**. The HMBC experiments [18, 19] were used advantageously in combining the individual assignments of the substructures of compounds **3** and **6** (established on the basis of the normal ^1H and ^{13}C NMR, ^1H-COSY, HSQC, DEPT-90 and 135



Scheme 2

experiments) into the corresponding full structures. The HMBC spectra were very helpful for locating the tertiary and quaternary carbons by identifying the various protons interacting with them through two-bond ($^2J_{CH}$), three-bond ($^3J_{CH}$) and occasionally four-bond ($^4J_{CH}$) couplings as displayed in Table 1.

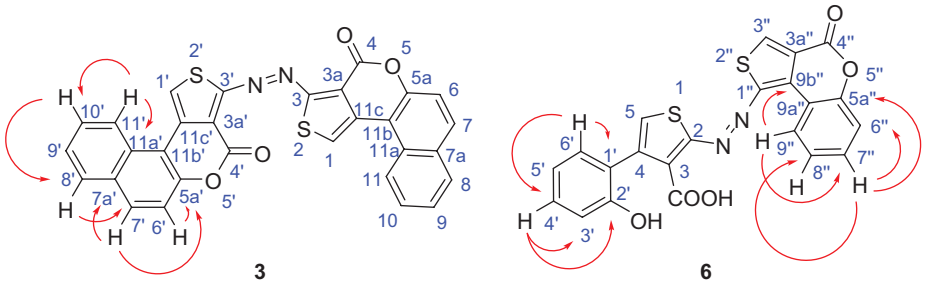
Similar analysis as above based on the available elemental and spectroscopic data was conducted for the full structural assignments of compounds **4–7**. Additionally, the presence of free phenolic OH groups in the hydrolysis products **5** and **6** was confirmed by positive ferric chloride phenol-enol tests [20, 21].

The complexity of the UV-visible spectra of these compounds is consistent with the presence of the diazo double bond (-N=N-) linking two fused thienocoumarin fragments and producing a highly conjugated system. This extended resonance is responsible for the bathochromic shifts of the n → π* and π → π* transitions of the parent chromophoric subsystems as well as those of the N=N double bond.

Biology

The azo compounds **3–7** and the substrates **1a** and **1b** were examined *in vitro* against bacterial and fungal species (Table 2). Results show that all microorganisms tested are differently inhibited by the azo dyes (MIC values between

Table 1 Important HMBC interactions and ^{1}H and ^{13}C chemical shifts δ in compounds **3** and **6** in DMSO-d_6 as the solvent (25°C).



Compound 3			Compound 6		
C atom	δ ^{13}C	HMBC (H → C)	C atom	δ ^{13}C	HMBC (H → C)
1	117.7	H-6 (7.61)	2	136.8	
3	155.3		3	154.4	
3a	115.3		4	148.2	
4	164.9		5	125.5	
5a	157.1	H-7 (8.38), H-6 (7.61)	1′	153.4	H-6′ (δ 7.90)
6	126.3		2′	154.5	H-4′ (δ 7.79)
7	138.1	H-8 (8.14)	3′	115.1	H-4′ (δ 7.79)
7a	131.6	H-11 (8.64), H-7 (8.38), H-9 (7.69), H-6 (7.61)	4′	117.9	H-6′ (δ 7.90)
8	130.5	H-10 (7.81), H-9 (7.69)	5′	120.0	
9	129.6		6′	129.0	
10	126.8	H-11 (8.64), H-6 (7.61)	COOH	184.9	
11	126.1	H-10 (7.81), H-9 (7.69)	1″	136.5	
11a	131.7		3″	116.8	
11b	113.8		3a″	154.2	
11c	102.0		4″	164.6	
			5a″	157.5	H-7″ (δ 7.76)
			6″	118.0	H-7″ (δ 7.76), H-8″ (δ 7.45)
			7″	128.9	
			8″	126.5	H-7″ (δ 7.76)
			9″	127.9	
			9a″	128.0	
			9b″	155.3	H-9″ (δ 8.65), H-8″ (δ 7.45)

Table 2 Minimum inhibitory concentration (MIC), minimum microbicidal concentration (MMC) and minimum fungicidal concentration (MFC) values (μg/mL) of compounds **1a,b** and **3–7** against bacterial and fungal strains.

Bacteria	Parameters	1a	1b	3	4	5	6	7	Reference drugs		
									Nys	Gen	Cipr
Shigella flexneri	MIC	256	256	–	2	32	16	8	nt	64	8
	MMC	>256	>256	–	4	32	16	16		64	8
Salmonella typhi	MIC	128	64	–	8	8	–	32	nt	32	8
	MMC	256	256	–	8	16	–	64		32	8
Staphylococcus aureus	MIC	256	64	256	2	8	64	32	nt	32	4
	MMC	>256	256	256	2	8	64	32		32	4
Cryptococcus neoformans	MIC	4	2	4	16	2	8	16	2	nt	nt
	MFC	8	4	16	16	2	8	16	2		
Candida glabrata	MIC	32	32	8	–	16	–	–	8	nt	nt
	MFC	128	128	64	–	16	–	–	8		
Candida albicans	MIC	128	128	32	32	4	8	64	2	nt	nt
	MFC	256	>256	32	64	8	8	128	2		

Nys, nystatin; Gen, gentamycin; Cipr, ciprofloxacin; nt, not tested; nd, not determined; –, not active.

2 and 256 μg/mL). The differences in the susceptibility of the microbial strains may be due to their structural and genetic variations. In general, except compound **3**, the transformations have improved the antibacterial activities of all the synthesized compounds. For the antifungal activities, the MIC values of the synthesized compounds are in some cases lower or equal to those of their precursors **1**. *Candida glabrata* and *Salmonella typhi* are the most resistant (MIC = 8–128 μg/mL) among yeasts and bacteria, respectively, whereas *Cryptococcus neoformans* is the most sensitive microorganism (MIC = 2–16 μg/mL). The antimicrobial activities of the tested compounds are in some cases equal or better than those of the reference drugs (nystatin, gentamicin and ciprofloxacin). Compound **5** is the most active substance against all microorganisms tested (MIC values between 2 and 32 μg/mL). It was also found that the MMC values obtained are generally less than fourfold greater than the MIC values (Table 2) on the corresponding microbial species, suggesting that a cidal effect of the tested samples can be expected on most of the tested microorganisms [22]. This is interesting from the perspective of developing new antimicrobial drugs from 3-aminothieno[3,4-*c*]coumarins. To our knowledge, this is the first report demonstrating the *in vitro* antimicrobial and antifungal activities of such compounds.

With regard to the structure-activity relationship analysis, compound **5** may be generated by hydrolysis of compound **4**. Compound **4** is the most active agent against bacteria species (MIC = 2–8 μg/mL), whereas compound **5** shows the best antifungal activity (MIC = 2–16 μg/mL). This analysis shows that the ring opening of the coumarins in **4** provides an increase in the antifungal activity of the resultant compound **5**. Similar results have been reported earlier [13, 23].

Conclusion

In this work, the challenge of bringing together thiophene, coumarin and diazo moieties into single molecular architectures has been successfully achieved. The hybrid compounds were found to possess rather interesting antibacterial and antifungal activities against selected yeast and bacterial strains. This study clearly demonstrates that the screened compounds are potential drug candidates in the treatment of microbial infections.

Experimental

General

All melting points are corrected and were determined with an Electrothermal Melting Point Apparatus Model 9100 and a Büchi 530 melting point apparatus. The thin layer chromatography (TLC) analyses were carried out on Eastman Chromatogram Silica Gel Sheets with a fluorescent indicator. A mixture of ethyl acetate and methylene chloride (7:3) was used as eluent, and iodine was used to visualize the separations. The IR spectra were measured with a Fourier Transform Infrared spectrometer JASCO FT/IR-4100 and a Perkin Elmer FT-IR

2000 spectrometer in KBr pellets. The UV-vis spectra were recorded in tetrahydrofuran (THF) using a Beckman U-640 spectrophotometer. Combustion analyses were carried out on a CHNS Euro analyzer. EI mass spectra (70 eV) were measured on a LCQ Classic spectrometer from Thermo Fisher Scientific Company. ^{1}H NMR spectra (750 MHz) and ^{13}C NMR spectra (187.5 MHz) were recorded in DMSO-d_6 on a Bruker AV III instrument. All reagents were purchased from Aldrich and Fluka and were used without further purification. Starting materials **1a–c** were prepared according to literature procedures as published earlier [11, 12].

General procedure for the preparation of the coupling products 3–7

Dry sodium nitrite (2.07 g, 3 mmol) was slowly added over a period of 30 min to concentrated sulfuric acid (10 mL) with occasional stirring. The solution was cooled to 0–5°C. Compound **1a,b** was dissolved in DMSO (10 mL) and the solution cooled to 0–5°C. The nitrosyl sulfuric acid solution was added to the solution of **1** while the temperature was maintained at 0–5°C. The clear diazonium salt solution **2a,b** thus generated was used immediately in the coupling reactions.

Substituted 2-aminothiophene derivative **1a–c** (3 mmol) was dissolved in DMSO (10 mL) and the solution cooled in an ice bath to 0–5°C. The diazonium solution, generated as described above, was added dropwise over 1 h, and then the mixture was treated with 15 mL of sodium acetate solution (10%). The resultant precipitate was collected and crystallized from methanol.

3-[2-(4-Oxo-4*H*-benzo[*f*]thieno[3,4-c]chromen-3-yl)diazenyl]-4*H*-benzo[*f*]thieno[3,4-*c*]chromen-4-one sulfate monohydrate (3) Reaction of **1a** with the diazonium reagent **2a** gave compound **3** (440 mg, 45%) as red powder; mp 194°C (dec); ^{1}H NMR: δ 7.60 (s, 2H, 1-H), 7.61 (d, 2H, *J* = 9.0 Hz, 6-H), 8.38 (d, 2H, *J* = 9.0 Hz, 7-H), 8.14 (d, 2H, *J* = 7.5 Hz, 8-H), 7.69 (dd, 1H, *J* = 7.0 and 7.5 Hz, 9-H), 7.81 (dd, 2H, *J* = 7.0 and 8.0 Hz, 10-H), 8.65 (d, 2H, *J* = 9.0 Hz, 11-H); ^{13}C NMR: δ (see Table 1); UV-vis: λ_{max} 242, 256, 291, 335, 345, 359, 374, 389 nm; IR: $\bar{\nu}$ 3300, 3400 (=NH), 1717 (C=O), 1615, 1597, 1440 (N=N) cm^{-1}; MS: m/z (%) 276 (13), 288 (33), 296 (100), 373 (60), 393 (53), 490 (10), 545 (1), 618 (1), 646 (1). Anal. Calcd for $C_{30}H_{18}N_2O_9S_3$: C, 55.72; H, 2.81; N, 4.33; S, 14.88. Found: C, 55.71; H, 2.79; N, 4.34; S, 14.89. Rf: 0.58.

3-[2-(3-amino-4-oxo-4H-thieno[3,4-c]chromen-1-yl)diazenyl]-4H-benzo[f]thieno[3,4-c]chromen-4-one disulfate (4) Reaction of **1c** with the diazonium solution **2a** gave compound **4** (420 mg, 30%) as green powder; mp 191°C (dec); ^{1}H NMR: δ 7.50 (s, 1H, 1-H), 7.62 (d, 1H, *J* = 9.0 Hz, 6-H), 8.38 (d, 1H, *J* = 9.0 Hz, 7-H), 7.80 (d, 1H, *J* = 7.0 Hz, 8-H), 7.70 (dd, 1H, *J* = 6.0 and 7.0 Hz, 9-H), 7.55 (dd, 1H, *J* = 6.0 and 7.0 Hz, 10-H), 8.65 (d, 1H, *J* = 8.0 Hz, 11-H), 7.54 (d, 1H, *J* = 7.0 Hz, 6′-H), 7.86 (dd, 1H, *J* = 7.0 and 7.0 Hz, 7′-H), 8.72 (dd, 1H, *J* = 8.0 and 8.0 Hz, 8′-H), 8.13 (d, 1H, *J* = 8.0 Hz, 9′-H), 8.08 (D_2O-exchangeable, NH_2); ^{13}C NMR: δ 120.1 (C-1), 135.5 (C-3 and C-1′), 104.9 (C-3a), 101.5 (C-3a′), 164.5 (C-4 and C-4′), 154.8 (C-5a), 154.9 (C-5a′), 125.6 (C-6 and C-6′), 126.3 (C-7), 131.1 (C-7a and C-11b), 129.9 (C-8), 117.1 (C-11), 129.1 (C-9), 125.4 (C-10 and C-8′), 137.6 (C-11a), 113.3 (C-11c), 156.6 (C-3′), 117.2 (C-7′), 128.9 (C-9′), 114.8 (C-9a′), 147.8 (C-9b′); UV-vis: λ_{max} 242, 248, 256.5, 278, 293.5, 334, 346, 361.5, 373.5, 389.5 nm; IR: $\bar{\nu}$ 3639, 3542, 3494, 3271 (=NH, NH_2), 3109, 3002 (Ar, C-H), 2794, 1730 (C=O), 1703, 1618, 1529, 1442 (N=N) cm^{-1}; MS: m/z (%) 313 (13), 347 (10), 493 (100), 414 (1), 444 (1), 494 (26), 495 (14), 555 (6), 617 (6). Anal. Calcd for $C_{26}H_{17}N_3O_{12}S_4$: C, 45.15; H, 2.48; N, 6.08; S, 18.54. Found: C, 45.13; H, 2.46; N, 6.07; S, 18.43. Rf: 0.55.

2-Amino-5-{2-[3-carboxy-4-(2-hydroxy-1-naphthyl)-2-thienyl] diazenyl}-4-(2-hydroxyphenyl)-3-thiophenecarboxylic acid sulfate (5) Reaction of **1b** with the diazonium solution **2a** gave compound **5** (640 mg, 51%) as brown powder; mp 140°C (dec); ^{1}H NMR: δ 7.32 (d, 1H, *J* = 7.5 Hz, 3′-H), 7.45 (dd, 1H, *J* = 7.0 and 7.5 Hz, 4′-H), 7.32 (dd, 1H, *J* = 7.0 and 7.0 Hz, 5′-H), 8.23 (d, 1H, *J* = 7.0 Hz, 6′-H), 7.11 (s, 1H, 5″-H), 7.54 (d, 1H, *J* = 9.0 Hz, 3‴-H), 8.17 (d, 1H, *J* = 9.0 Hz, 4‴-H), 8.09 (d, 1H, *J* = 8.0 Hz, 5‴-H), 7.49 (dd, 1H, *J* = 7.0 and 7.5 Hz, 6‴-H), 7.70 (dd, 1H, *J* = 7.5 and 8.0 Hz, 7‴-H), 8.52 (d, 1H, *J* = 8.0 Hz, 8‴-H), 8.08 (D_2O-exchangeable, NH_2); ^{13}C NMR: δ 154.2 (C-2), 119.5 (C-3), 153.4 (C-4), 136.3 (C-5), 152.9 (C-1′), 154.5 (C-2′), 116.0 (C-3′), 117.3 (C-4′), 120.2 (C-5′), 127.9 (C-6′), 184.3 (2 × COOH), 133.2 (C-2″), 116.2 (C-3″), 102.9 (C-4″), 118.1 (C-5″), 129.5 (C-1‴), 156.4 (C-2‴), 125.5 (C-3‴), 138.9 (C-4‴), 130.9 (C-4a‴), 129.5 (C-5‴), 117.2 (C-6‴), 129.9 (C-7‴), 122.9 (C-8‴), 137.6 (C-8a‴); IR: $\bar{\nu}$ 1716 (C=O), 1532, 1440 (N=N) cm^{-1}; MS: m/z (%) 219 (6), 288 (35), 296 (100), 316 (9), 372 (25), 385 (36), 393 (65), 394 (5), 490 (4). Anal. Calcd for $C_{26}H_{19}N_3O_{10}S_3$: C, 49.60; H, 3.04; N, 6.67; S, 15.28. Found: C, 49.57; H, 3.02; N, 6.65; S, 15.26. Rf: 0.77.

2-[2-(3-Acetyl-4-hydroxyphenyl)diazenyl]-4-(2-hydroxyphenyl)-3-thiophene carboxylic acid (6) Reaction of **1b** with the diazonium solution **2b** gave compound **6** (560 mg, 41%) as yellow powder; mp 187°C (dec); ^{1}H NMR: δ 7.43 (s, 2H, 5-H and 3″-H), 7.43 (d, 1H, *J* = 7.0 Hz, 3′-H), 7.79 (dd, 1H, *J* = 7.0 and 8.5 Hz, 4′-H), 7.45 (dd, 2H, *J* = 8.0 and 8.5 Hz, 5′-H and 8″-H), 7.90 (d, 1H, *J* = 8.0 Hz, 6′-H), 7.41 (d, 1H, *J* = 8.5 Hz, 6″-H), 7.76 (dd, 1H, *J* = 8.0 and 8.5 Hz, 7″-H), 8.65 (d, 1H, *J* = 8.0 Hz, 9″-H); ^{13}C NMR: δ (see Table 1); UV-vis: λ_{max} 241, 251, 263.5, 299, 313, 327.5, 384.5 nm; IR: $\bar{\nu}$ 3540, 3288 (OH), 1724 (C=O), 1678, 1447 (N=N) cm^{-1}; MS: m/z (%) 194 (6), 225 (12), 247 (100), 307 (33), 310 (12), 382 (9), 390 (20), 406 (1). Anal. Calcd for $C_{22}H_{12}N_2O_5S_2$: C, 58.92; H, 2.70; N, 6.25; S, 14.30. Found: C, 58.90; H, 2.68; N, 6.26; S, 14.31. Rf: 0.53.

3-Amino-1-[2-(4-oxo-4*H*-thieno[3,4-c]chromen-3-yl)diazenyl]-7,11*b*-dihydro-4*H*-benzo[*f*]thieno[3,4-*c*]chromen-4-one (7) Reaction of **1a** with the diazonium solution **2b** gave compound **7** (670 mg, 45%) as green powder; mp 230°C (dec); ^{1}H NMR: δ 7.50 (d, 1H, *J* = 8.0 Hz, 6-H), 8.13 (d, 1H, *J* = 8.0 Hz, 7-H), 8.70 (d, 1H, *J* = 8.0 Hz, 8-H), 8.31 (dd, 1H, *J* = 6.0 and 8.0 Hz, 9-H), 7.22 (dd, 1H, *J* = 6.0 and 7.5 Hz, 10-H), 8.75 (d, 1H, *J* = 7.5 Hz, 11-H), 7.68 (D_2O-exchangeable, NH_2), 7.60 (s, 1H, 1′-H), 7.72 (d, 1H, *J* = 9.2 Hz, 6′-H), 7.83 (ddd, 1H, *J* = 1.5, 8.0 and 10.0 Hz, 7′-H), 7.75 (ddd, 1H, *J* = 1.5, 9.2 and 10.0 Hz, 8′-H), 7.40 (d, 1H, *J* = 10.0 Hz, 9′-H); ^{13}C NMR: δ 133.4 (C-1), 157.8 (C-3), 115.2 (C-3a), 164.8 (C-4), 155.6 (C-5a), 126.4 (C-6), 139.9 (C-7), 133.2 (C-7a), 129.6 (C-8), 119.4 (C-9), 127.7 (C-10), 124.9 (C-11), 130.8 (C-11a), 130.7 (C-11b), 103.7 (C-11c), 117.4 (C-1′), 135.4 (C-3′), 154.2 (C-3a′), 163.6 (C-4′), 152.7 (C-5a′), 118.1 (C-6′), 128.2 (C-7′), 124.9 (C-8′), 127.2 (C-9′), 130.8 (C-9a′), 155.4 (C-9b′); UV-vis: λ_{max} 242.5, 255, 277.5, 290, 297, 335.5, 344.5, 360, 374.5, 390 nm; IR: $\bar{\nu}$ 1720 (C=O), 1617, 1439 (N=N) cm^{-1}; MS: m/z 211 (12), 219 (34), 236 (27), 268 (100), 288 (25), 296 (54), 312 (12), 338 (32), 360 (12), 392 (11), 447 (1). Anal. Calcd for $C_{26}H_{13}N_3O_4S_2$: C, 63.02; H, 2.64; N, 8.48; S, 12.94. Found: C, 63.00; H, 2.62; N, 8.46; S, 12.95. Rf: 0.56.

Biological assay

Microorganisms The microorganisms used in this study consisted of two bacteria (*Staphylococcus aureus* ATCC25922 and *Salmonella typhi* ATCC6539) and one *Candida* species (*Candida albicans* ATCC 9002); all of which are reference strains obtained from the American Type Culture Collection. Also, included were one clinical isolate of *Shigella flexneri* collected from the Pasteur Centre (Yaounde, Republic of Cameroon) and two strains of *Cryptococcus neoformans* IP95026 and *Candida glabrata* IP35 obtained from the Pasteur Institute (IP, Paris, France). The bacterial and yeast strains were grown at 35°C and maintained on nutrient agar (NA, Conda, Madrid, Spain) and Sabouraud Dextrose Agar (SDA, Conda) slants, respectively.

Determination of minimum inhibitory concentration (MIC) and minimum microbicidal concentration (MMC) MIC values were determined by a broth micro-dilution method as described earlier [24] with slight modifications. Each test sample was dissolved in dimethylsulfoxide (DMSO) and the solution was then added to Mueller Hinton Broth (MHB) for bacteria or Sabouraud Dextrose Broth (SDB) for yeasts to give a final concentration of 1024 μg/mL. This was serially diluted twofold to obtain a concentration range of 0.50–1024 μg/mL. Then, 100 μL of each concentration was added in each well (96-well microplate) containing 95 μL of MHB or SDB and 5 μL of inoculum for final concentrations varying from 0.25–512 μg/mL. The inoculum was standardized at 1.5×10^6 CFU/mL by adjusting the optical density to 0.1 at 600 nm using a JENWAY 6105 UV/Vis spectrophotometer. The final concentration of DMSO in each well was <1% [preliminary analyses with 1% (v/v) DMSO did not inhibit the growth of the test organisms]. The negative control well consisted of 195 μL of MHB or SDB and 5 μL of the standard inoculum. The plates were covered with sterile lids, then agitated to mix the contents of the wells using a plate shaker and incubated at 35°C for 24 h (for bacteria) or for 48 h (for yeasts). The assay was repeated three times. The MIC values of samples were determined by adding 50 μL of a 0.2 mg/mL *p*-iodonitrotetrazolium violet solution followed by incubation at 35°C for 30 min. Viable microorganisms reduced the yellow dye to a pink color. MIC values were defined as the lowest sample concentrations that prevented this change in color indicating a complete inhibition of microbial growth.

For the determination of MMC values, a portion of liquid (5 μL) from each well that showed no growth of microorganism was plated on Mueller Hinton Agar or SDA and incubated at 35°C for 24 h (for bacteria) or 35°C for 48 h (for yeasts). The lowest concentrations that yielded no growth after this subculturing were taken as the MMC values [24]. Ciprofloxacin (Sigma-Aldrich, Steinheim, Germany) and nystatin (Merck, Darmstadt, Germany) were used as positive controls for bacteria and yeasts, respectively.

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