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.

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 diazotization by the nitrosyl sulfuric acid used in excess. By contrast, the in situ deaminated [16, 17] intermediate 2b is generated.

The thienylidiazonium 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...
product 3A most probably undergoes coupling with the thienydiazonium 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 thienydiazonium 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 H₂SO₄ and 1 molecule of H₂O. Structure 3 was assigned on the basis of the combustion analysis data which established the formula to be C₃₀H₁₈N₂O₉S₃. In the IR spectrum, the characteristic N-H stretching frequencies of the NH₂ 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 ¹³C 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 ¹³C NMR, ¹H-COSY, HSQC, DEPT-90 and 135
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 ($J_{CH}$), 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 $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ 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...
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.

### 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.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Parameters</th>
<th>1a</th>
<th>1b</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>Reference drugs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shigella flexneri</td>
<td>MIC</td>
<td>256</td>
<td>256</td>
<td>–</td>
<td>2</td>
<td>32</td>
<td>16</td>
<td>8</td>
<td>nt</td>
</tr>
<tr>
<td></td>
<td>MMC</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>–</td>
<td>4</td>
<td>32</td>
<td>16</td>
<td>16</td>
<td>nt</td>
</tr>
<tr>
<td>Salmonella typhi</td>
<td>MIC</td>
<td>128</td>
<td>64</td>
<td>–</td>
<td>8</td>
<td>8</td>
<td>–</td>
<td>32</td>
<td>nt</td>
</tr>
<tr>
<td></td>
<td>MMC</td>
<td>256</td>
<td>256</td>
<td>–</td>
<td>8</td>
<td>16</td>
<td>–</td>
<td>64</td>
<td>32</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>MIC</td>
<td>256</td>
<td>64</td>
<td>256</td>
<td>2</td>
<td>8</td>
<td>64</td>
<td>32</td>
<td>nt</td>
</tr>
<tr>
<td></td>
<td>MMC</td>
<td>&gt;256</td>
<td>64</td>
<td>256</td>
<td>2</td>
<td>8</td>
<td>64</td>
<td>32</td>
<td>4</td>
</tr>
<tr>
<td>Cryptococcus neoformans</td>
<td>MIC</td>
<td>4</td>
<td>2</td>
<td>4</td>
<td>16</td>
<td>2</td>
<td>8</td>
<td>16</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>MFC</td>
<td>8</td>
<td>4</td>
<td>16</td>
<td>16</td>
<td>2</td>
<td>8</td>
<td>16</td>
<td>2</td>
</tr>
<tr>
<td>Candida glabrata</td>
<td>MIC</td>
<td>32</td>
<td>32</td>
<td>8</td>
<td>–</td>
<td>16</td>
<td>–</td>
<td>–</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>MFC</td>
<td>128</td>
<td>128</td>
<td>64</td>
<td>–</td>
<td>16</td>
<td>–</td>
<td>–</td>
<td>8</td>
</tr>
<tr>
<td>Candida albicans</td>
<td>MIC</td>
<td>128</td>
<td>128</td>
<td>32</td>
<td>32</td>
<td>4</td>
<td>8</td>
<td>64</td>
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</tr>
<tr>
<td></td>
<td>MFC</td>
<td>256</td>
<td>&gt;256</td>
<td>32</td>
<td>64</td>
<td>8</td>
<td>8</td>
<td>128</td>
<td>2</td>
</tr>
</tbody>
</table>

Nys, nystatin; Gen, gentamycin; Cipr, ciprofloxacin; nt, not tested; nd, not determined; –, not active.
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, described as prepared 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-4H-benzo[thieno[3,4-c]chromen-3-yl)diazeyl]-4H-benzo[thieno[3,4-c]chromen-4-one sullfate monohydrate (3) Reaction of 1a with the diazonium reagent 2a gave compound 3 (440 mg, 45%) as red powder; mp 194°C (dec); ¹H 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, 2H, 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). ¹C NMR: δ see (Table I); UV-vis: 242, 256, 291, 334, 359, 374, 389, 394 nm; IR: v 3300, 3400, 1717 (C=O), 1615, 1597, 1440 (N−C) cm−1; MS: m/z (%): 242 (6), 225 (12), 247 (100), 307 (36), 385 (36), 393 (65), 394 (50), 490 (4). Anal. Calcd for C₂₅H₁₇N₃O₁₂S₄: C, 58.92; H, 2.70; N, 6.25; S, 14.30. Found: C, 58.90; H, 2.68; N, 6.62; S, 14.31. RF: 0.53.

3-[2-(3-Acetyl-4-hydroxyphenyl)diazeyl]-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); ¹H NMR: δ 7.43 (s, 2H, 5-H and 6-H), 7.43 (d, 1H, J = 7.0 Hz, 7-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). ¹C NMR: δ (see Table I); UV-vis: v 3540, 3288 (OH), 1724 (C=O), 1678, 1447 (N−N) cm−1; MS: m/z (%): 194 (6), 225 (12), 247 (24), 307 (100), 337 (33), 310 (12), 382 (9), 390 (20), 406 (1). Anal. Calcd for C₂₆H₁₉N₃O₁₀S₃: C, 58.92; H, 2.70; N, 6.25; S, 14.30. Found: C, 58.90; H, 2.68; N, 6.62; S, 14.31. RF: 0.53.

3-Amino-1-[2-(4-oxo-4H-thieno[3,4-c]chromen-3-yl)diazeyl]-7-bromo-4H-benzo[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); ¹H 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), 6.78 (d, 1H, J = 8.0 Hz, 1′-H), 7.27 (dd, 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). ¹C NMR: δ 133.4 (C-4), 157.8 (C-5), 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), 124.9 (C-10), 130.8 (C-11a), 130.1 (C-11b), 103.7 (C-11c), 117.4 (C-1′), 135.4 (C-2′), 154.2 (C-3′), 163.6 (C-4′), 152.7 (C-5′a), 118.1 (C-6′), 128.2 (C-7′), 124.9 (C-8′), 127.2 (C-9′), 130.8 (C-9′a), 154.9 (C-9′b). UV-vis: λmax: 242, 255, 277, 290, 297, 335, 345, 360, 374, 395 nm; IR: v 3390 (OH), 1720 (C=O), 1617, 1439 (N−N) cm−1; MS: m/z (%): 212 (11), 219 (3a), 236 (27), 268 (100), 288 (25), 296 (54), 312 (32), 338 (32), 360 (12), 392 (11), 447 (1). Anal. Calcd for C₂₆H₁₇N₃O₁₂S₂: 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 (Yaoundé, 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 0.5–1024 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 × 10^5 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 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 [26]. Ciprofloxacin (Sigma-Aldrich, Steinheim, Germany) and nystatin (Merck, Darmstadt, Germany) were used as positive controls for bacteria and yeasts, respectively.

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