**ORIGINAL ARTICLE**

**New 3-substituted-2,1-benzisoxazoles: Synthesis and antimicrobial activities**

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Received 22 May 2013; accepted 7 September 2013

**KEYWORDS**

2,1-Benzisoxazole; Anthranil; Antiplasmodial; Antimicrobial

**Abstract** A new series of 3-substituted-2,1-benzisoxazoles (anthranils) were prepared by different methods and characterized by spectroscopic methods and mass spectrometry. These 2,1-benzisoxazoles were tested *in vitro* for their antiplasmodial activity on a chloroquine-resistant strain of *Plasmodium falciparum* (*P*f.,) (*FcB1*), and for antimicrobial activity against representative bacterial and fungal strains, as well as for cytotoxicity on MCF7 human breast cancer cells. Given the log \(P_{\text{calc}}\) and selectivity index values (cytotoxicity/antiplasmodial activity ratio), the benzo[\(c\)]isoxazol-3-ylmethylene-phenyl-amine (11) (imino-benzisoxazole) was identified as the best hit against *P*f., (*FcB1*), and the benzo[\(c\)]isoxazol-3-yl-phenyl-methanone (3) (3-acyl-2,1-benzisoxazole) against *P*f. and the *Geotrichum candidum* fungal strain.

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1. Introduction

The interest in heterocyclic compounds is basically because of their biological activities and pharmacological properties.

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http://dx.doi.org/10.1016/j.arabjc.2013.09.011

Please cite this article in press as: Chaker, A. et al., New 3-substituted-2,1-benzisoxazoles: Synthesis and antimicrobial activities. Arabian Journal of Chemistry (2013), http://dx.doi.org/10.1016/j.arabjc.2013.09.011
Figure 1  Structure of 3-substituted-2,1-benzisoxazoles 1–13.

<table>
<thead>
<tr>
<th>Compd.</th>
<th>Structure</th>
<th>M (g mol⁻¹)/Log P&lt;sub&gt;calc&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt;</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (µmol L⁻¹) FcB1</th>
<th>CC&lt;sub&gt;50&lt;/sub&gt; (µmol L⁻¹)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>MCF7</th>
<th>Selectivity index&lt;sup&gt;c&lt;/sup&gt; MCF-7/FcB1</th>
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<tr>
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<td>Sodium artemisate</td>
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<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Log P<sub>calc</sub> calculated with VCCLAB (http://www.virtualaboratory.org/lab/alogps/start.html).

<sup>b</sup> The drug concentration needed to cause a 50% decrease in cell viability; The IC<sub>50</sub> SD were always lower than 20% and were discarded for maximum lisibility.

<sup>c</sup> Cytotoxicity/antiplasmodial activity ratio.
heterocyclic systems, for instance, into acridinones (Ogata et al., 1969). The acridinones are used extensively in the production of dyes and pharmaceuticals (Ogata et al., 1969), and also as biologically active compounds acting as anti-inflammatory agents (Walsh, 1987) and glycohydrolase inhibitors (Farr and Peet, 1992). We were interested by the wide variety of pharmacological properties of benzisoxazoles and so we designed the synthesis of a new series of 3-substituted-2,1-benzisoxazole in a continuing search for new alternative drugs for malaria and infectious diseases (Nepveu et al., 2010; Najahi et al., 2013). In this paper we report the antiplasmodial and antimicrobial activities of a series of 3-substituted-2,1-benzisoxazoles 1–13 (See Fig. 1).

2. Results and discussion

2.1. Chemistry

The series of 3-substituted-2,1-benzisoxazoles (anthranils) (Table 1) were prepared from 2,1-benzisoxazole-3-carboxylic acid 1 (Eckroth and Cochran, 1970) and 3-amino-2,1-benzisoxazole 2 (Musso and Schroeder, 1965) (Fig. 2). Different synthetic pathways were considered and successfully used in this work, which led to the desired 3-substituted-2,1-benzisoxazoles. Scheme 1 shows the synthesis of anthranils starting from 2,1-benzisoxazole-3-carboxylic acid 1. The Friedel-Crafts reaction of the 2,1-benzisoxazole-3-carboxylic acid 1 with benzene or substituted benzene (chlorobenzene, anisole or toluene) in dry benzene (96–98%) in the presence of oxalyl chloride and aluminium chloride as catalyst yielded the expected 2,1-benzisoxazoles of type 3 (Benjamin and Theodorus, 1993; Benjamin et al., 1991) or (4–6) in acceptable yields (45–61%).

The 2,1-benzisoxazoles 7–9 were prepared starting from compound 1 with aniline, diethylamine and 4-methoxyphenylamine, respectively, in the presence of oxalyl chloride in anhydrous toluene in low yield (Scheme 1).

The reduction of the 2,1-benzisoxazole 1 via the benzylic alcohol provided the anthranil 10 (Jackie et al., 2007), which was transformed to the 3-imino-2,1-benzisoxazole 11 in an acceptable yield by condensation with aniline (Scheme 2).

The 3-amino-2,1-benzisoxazole 2 condensed with benzyl chloride in a solution of toluene and triethylamine gave the anthranil 12 at a 22% yield. Approximately 7% of the compound 2 was converted to the 2,1-benzisoxazole 13 as determined by 1H NMR of the crude material (Scheme 3).

The lipophilicity of the synthesized derivatives 1–12 was expressed in terms of their Log $P_{\text{calc}}$ values that were calculated with the VCCLAB software. As shown in Table 1, higher Log $P_{\text{calc}}$ values were observed for compounds 3–7 and 11–12 (3.11–3.69), compared to the 2,1-benzisoxazoles 1, 2 and 10 (1.36–1.49). For the former, the values were comparable to those of the antimalarials sodium artesunate and of chloroquine.

2.2. Pharmacology

2.2.1. Antiplasmodial and cytotoxic activities

Antiplasmodial and cytotoxic activities were performed as previously described (Nepveu et al., 2010; Najahi et al., 2013). The
antiplasmodial activities of the 2,1-benzisoxazoles 1–12 expressed as the IC\textsubscript{50} values, ranged between 12.4 and 130.6 \textmu mol L\textsuperscript{-1} \textsuperscript{1} (Table 1). The 2,1-benzisoxazole-3-carboxylic acid 1 where R\textsuperscript{3} = -COOH had the weakest activities with IC\textsubscript{50} values higher than 60 \textmu mol L\textsuperscript{-1}. Groups with higher lipophilicity (log P > 3) at position R\textsuperscript{3} (acyl/amide/imine) improved the antiplasmodial activity (IC\textsubscript{50}/\textmu mol L\textsuperscript{-1} \textsuperscript{1} \approx 12–16) (3–6, 11) while groups (aldehyde) at R\textsuperscript{3} with lower lipophilicity (log P < 3) led to a significant decrease in the activity (IC\textsubscript{50}/\textmu mol L\textsuperscript{-1}: 10 = 68). Fig. 3 illustrates the inverse relation between Log P\textsubscript{calc} and the IC\textsubscript{50} values.

Cytotoxicity was assayed on the mammalian MCF7 cell line and was higher than 15 \textmu mol L\textsuperscript{-1} \textsuperscript{1} with CC\textsubscript{50} values ranging from 15.8 to 680 \textmu mol L\textsuperscript{-1} \textsuperscript{1} (Table 1).

2.2.2. In vitro evaluation of antimicrobial activity

The synthesized 2,1-benzisoxazoles 1–12 were evaluated for their \textit{in vitro} antibacterial activity against representative Gram-positive (\textit{Bacillus subtilis}, \textit{Staphylococcus aureus}) and Gram-negative bacteria (\textit{Pseudomonas aeruginosa}, \textit{Cronobacter sakazakii}) and against two fungi (\textit{Geotrichum candidum}, \textit{Candida albicans}) using reported techniques (Najahi et al., 2013). Minimum inhibitory concentrations (MICs) were calculated in order to express the antimicrobial activity and were defined as the concentration of the compound required to give complete inhibition of the bacterial growth. From the 12 tested compounds, only three presented antimicrobial activities. Their MICs values are reported in Table 2 with those of reference drugs. The best activities were observed for compound 3.

<table>
<thead>
<tr>
<th>Compound</th>
<th>\textit{Pseudomonas aeruginosa}</th>
<th>\textit{Cronobacter sakazakii}</th>
<th>\textit{Bacillus subtilis}</th>
<th>\textit{Staphylococcus aureus}</th>
<th>\textit{Geotrichum candidum}</th>
<th>\textit{Candida albicans}</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>MIC\textsuperscript{a}</td>
<td>MIC\textsuperscript{b}</td>
<td>MIC\textsuperscript{b}</td>
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<td>b</td>
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<td>b</td>
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<tr>
<td>12</td>
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<td>b</td>
<td>b</td>
<td>b</td>
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<td>17.9</td>
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<td>2.3</td>
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<td>b</td>
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<tr>
<td>Ampicillin</td>
<td>71.9</td>
<td>14.3</td>
<td>14.5</td>
<td>0.6</td>
<td>b</td>
<td>b</td>
</tr>
<tr>
<td>Amphotericin\textsuperscript{c}</td>
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<td>b</td>
<td>b</td>
<td>6.8</td>
<td>0.3</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a} MICs: minimum inhibitory concentration (\textmu mol L\textsuperscript{-1}).  
\textsuperscript{b} Inactive.  
\textsuperscript{c} Ref. (Sharma et al., 2011).
New 3-substituted-2,1-benzisoxazoles: Synthesis and antimicrobial activities

(3. Conclusions

We report a new approach to the synthesis of 3-substituted-2,1-benzisoxazoles starting from 2,1-benzisoxazole-3-carboxylic acid and 3-amino-2,1-benzisoxazole. Although representatives of this new series do not exhibit strong antimicrobial activities, it should be noted that compound 11, with an IC50 value around the 10 μmol level against P. f., had however the best selectivity index in the series. This was achieved without any substituent effect on either ring. Thus, there are actually opportunities of pharmaco-modulation for this compound by introducing groups on both rings to try to reach an IC50 value in the nanomolar range and then multiplying the selectivity index by a factor 102. Compounds 3-6 also showed interesting antiplasmodial activities with an initial small pharmaco-modulation effect of the substituent in the para position of the phenyl ring. Working on both rings by introducing new substituents may give good to very good antiplasmodial activities. Compound 3 gave a good result on G. candidum but other similar derivatives (4-6) were inactive leaving little scope for pharmacological modulation.

4. Experimental

4.1. General

Commercially available reagent grade chemicals were used as received without additional purification. All reactions were followed by TLC (E. Merck Kieselgel 60 F-254), with detection by UV light at 254 nm. Column chromatography was performed on silica gel (60–200 mesh E. Merck). IR spectra were recorded on a Perkin-Elmer PARAGON 1000 FT-IR spectrometer. 1H and 13C NMR spectra were recorded on an AC Bruker spectrometer at 300 MHz (1H) and 75 MHz (13C) using (CD3)2SO as solvent with (CD3)2SO (δH 2.5) or (CD3)2SO (δC 39.5). Chemical shifts (δ) are reported in parts per million (ppm) relative to tetramethylsilane (0 ppm). High-resolution mass spectra (DCI in CH4) were recorded on a Bruker Maxis spectrometer (Service Commun Toulouse, France). Melting points were determined with an Electrotherm 9300 capillary melting point apparatus and are uncorrected. The purity of all compounds was determined by LC-PDA–MS methods and was found to be in the range 96–99%.

4.2. Synthesis of 2,1-benzisoxazoles 3–6

This procedure was used for compounds 3-6. For example, for compound 3 oxalyl chloride (m = 2.5 g, 20 mmol) was added to benzisoxazole-3-carboxylic acid (0.150 g, 1 mmol) in dry benzene (4 mL), and the reaction mixture was stirred at 80 °C under a nitrogen atmosphere for 4 h. The solvent was vacuum evaporated and the resultant residue dissolved in dry benzene (4 mL), and AlCl3 (0.392 g, 2.94 mmol) was added and the reaction mixture stirred at 80 °C for 1 h. This reaction mixture was then poured into ice-cold water and 37% aqueous HCl and extracted with ethyl acetate (3 × 100 mL) and the combined organic phase subsequently dried over anhydrous MgSO4 and vacuum evaporated to afford the crude product. This was further purified by column chromatography using ethyl acetate-petroleum ether (v/v = 20/80) and recrystallized from methanol-H2O (v/v = 60/40).

4.2.1. Benzof[c]isoxazol-3-yl-phenyl-methanone (3)

Yellow powder, yield: 49%, mp: 96–98 °C. IR (KBr, cm–1): 1651, 1627, 1189. 1H NMR (300 MHz, DMSO-d6): δ: 8.14 (d, J = 6 Hz, 2H), 7.98 (d, J = 9 Hz, 1H), 7.91 (d, J = 9 Hz, 1H), 7.76 (d, J = 7.5 Hz, 1H), 7.60 (m, 3H), 7.44 (d, J = 7.8 Hz, 1H); 13C NMR (75 MHz, DMSO-d6): δ: 181.8 (C=O), 157.3 (C), 154.3 (C), 136.3 (C), 134.8 (CH), 132.5 (CH), 130.2 (2CH), 129.6 (CH), 129.4 (2CH), 121.4 (CH), 121 (C), 116.2 (CH). HRMS (DCI, CH3) m/z calced for C14H10NO2 [M + H]+ 224.0712. Found 224.0721.

4.2.2. Benzof[c]isoxazol-3-yl-(4-chloro-phenyl)-methanone (4)

Yellow powder, yield: 43%, mp: 127–129 °C. IR (KBr, cm–1): 1712, 1640, 1185, 753. 1H NMR (300 MHz, DMSO-d6): δ: 8.20 (d, J = 8.4 Hz, 2H), 8.02 (d, J = 8.7 Hz, 1H), 7.93 (d, J = 9 Hz, 1H), 7.76 (d, J = 8.7 Hz, 2H), 7.61 (dd, J = 9.3 Hz, J = 5.4 Hz, 1H), 7.45 (dd, J = 8.7 Hz, J = 6.6 Hz, 1H); 13C NMR (75 MHz, DMSO-d6): δC180 (C=O), 156.7 (C), 153.3 (C), 138.9 (C), 134.4 (CH3), 131.2 (1CH), 131.6 (2CH), 129.2 (CH), 129.2 (CH), 120.9 (CH), 120.6 (C), 115.7 (CH). HRMS (DCI, CH3) m/z calced for C14H10CINO2 [M + H]+ 258.0322. Found 258.0328.

4.2.3. Benzof[c]isoxazol-3-yl-(4-methoxy-phenyl)-methanone (5)

Yellow powder, yield: 13%, mp: 125–127 °C. IR (KBr, cm–1): 1637, 1600, 1240, 1179. 1H NMR (300 MHz, DMSO-d6): δ: 8.21 (d, J = 9 Hz, 2H), 8.01 (d, J = 8.4 Hz, 1H), 7.90 (d, J = 9 Hz, 1H), 7.57 (dd, J = 9 Hz, J = 6 Hz, 1H), 7.41 (dd, J = 8.7 Hz, J = 6.6 Hz, 1H), 7.21 (d, J = 9 Hz, 2H), 3.91 (s, 3H). HRMS (DCI, CH3) m/z calced for C14H12NO2 [M + H]+ 254.0817. Found 254.0826.

4.2.4. Benzof[c]isoxazol-3-yl-p-tolyl-methanone (6)

White powder, yield: 9%, mp: 105–107 °C. IR (KBr, cm–1): 1647, 1617, 1235. 1H NMR (300 MHz, DMSO-d6): δ: 8.10 (d, J = 8.4 Hz, 2H), 8.00 (d, J = 8.7 Hz, 1H), 7.92 (d, J = 9 Hz, 1H), 7.59 (dd, J = 9 Hz, J = 6.6 Hz, 1H), 7.44 (m, 3H), 2.46 (s, 3H). HRMS (DCI, CH3) m/z calced for C14H12NO2 [M + H]+ 238.0868. Found 238.0875.

4.3. Synthesis of 2,1-benzisoxazoles 7–9

This procedure was used for compounds 7–9. For example, for compound 7 oxalyl chloride (m = 2.5 g, 20 mmol) was added to benzisoxazole-3-carboxylic acid (0.150 g, 0.919 mmol) in dry toluene (4 mL), and the reaction mixture was stirred at 80 °C under nitrogen for 16 h. The solvent was vacuum evaporated and the resultant residue dissolved in dry toluene (2 mL). A solution of aniline (m = 0.207 g, 2.22 mmol) in dry toluene (4 mL), and AlCl3 (m = 2.5 g, 20 mmol) was added and the reaction mixture was stirred at 80 °C for 1 h. This reaction mixture was then poured into ice-cold water and 37% aqueous HCl and extracted with ethyl acetate (3 × 100 mL) and the combined organic phase subsequently dried over anhydrous MgSO4 and vacuum evaporated to afford the crude product. This was further purified by column chromatography using ethyl acetate-petroleum ether (v/v = 20/80) and recrystallized from methanol-H2O (v/v = 60/40).
was evaporated in vacuo and the dry residue was treated with aqueous NaHCO₃ (1 × 25 mL). The organic phase was washed with brine (1 mL) and dried over anhydrous MgSO₄ for 10 min and then benzoyl chloride (0.069 g, 0.492 mmol) was added drop-wise. The reaction mixture was stirred at 0–5 °C for 10 min and then benzoyl chloride (0.069 g, 0.492 mmol) was added drop-wise. The reaction mixture was then stirred at 0–5 °C for 2 h. The mixture was treated with 5% aqueous HCl (1 × 10 mL) and then extracted with ethyl acetate (3 × 25 mL), and the organic phase was filtered through anhydrous MgSO₄ and vacuum evaporated to afford the crude product. This was further purified by column chromatography using toluene-acetone (v/v = 5/95).

4.3.1. Benzo[c]isoxazole-3-carboxylic acid phenylamide (7)

Yellow powder, yield: 39%, mp: 161–163 °C. IR (KBr, cm⁻¹): 3270, 1676, 1617, 1152. ¹H NMR (300 MHz, DMSO-d₆): δ: 11.12 (s, 1H, NH), 8.03 (d, J = 9 Hz, 1H), 7.85 (m, 3H), 7.55 (dd, J = 9 Hz, J = 6.3 Hz, 1H), 7.36 (m, 3H), 7.18 (m, 1H). ¹³C NMR (75 MHz, DMSO-d₆): δ: 157.5 (C=–O), 157.2 (C), 154.9 (C), 132.5 (CH), 129.2 (2CH), 127.2 (CH), 125.1 (CH), 121.3 (CH), 121.2 (2CH), 119.3 (C), 115.7 (CH). HRMS (DCI, CH₃CN) m/z calcd for C₁₄H₁₁N₂O₂ [M+H]⁺ 239.0821. Found 239.0826.

4.3.2. Benzo[c]isoxazole-3-carboxylic acid diethylamide (8)

Yellow powder, yield: 34%, mp: 168–170 °C. IR (KBr, cm⁻¹): 3335, 1672, 1612, 1151. ¹H NMR (300 MHz, DMSO-d₆): δ: 7.83 (d, J = 8.7 Hz, 1H), 7.74 (d, J = 8.4 Hz, 1H), 7.49 (dd, J = 9 Hz, J = 6.3 Hz, 1H), 7.24 (dd, J = 8.7 Hz, J = 6.3 Hz, 1H), 3.56 (m, 4H), 1.23 (m, 6H). HRMS (DCI, CH₃CN) m/z calcd for C₁₂H₁₅N₂O₂ [M+H]⁺ 219.1134. Found 219.1121.

4.3.3. Benzo[c]isoxazole-3-carboxylic acid (4-methoxy-phenyl)-amide (9)

Yellow powder, yield: 27%, mp: 172–174 °C. IR (KBr, cm⁻¹): 3335, 1672, 1629, 1206, 1189. ¹H NMR (300 MHz, DMSO-d₆): δ: 11.01 (s, 1H, NH), 8.02 (d, J = 9 Hz, 1H), 7.79 (m, 3H), 7.55 (dd, J = 9 Hz, J = 6.3 Hz, 1H), 7.43 (d, J = 8.7 Hz, 6.6 Hz, 1H), 6.97 (d, J = 9 Hz, 2H), 3.76 (m, 3H). ¹³C NMR (75 MHz, DMSO-d₆): δ: 157 (C=–O), 156.9 (C), 156.1 (C), 154.1 (C), 132 (CH), 130.8 (C), 127.2 (CH), 122.2 (2CH), 120.9 (CH), 118.7 (C), 115.1 (CH), 113.8 (2CH), 55.2 (OCH₃). HRMS (DCI, CH₃CN) m/z calcd for C₁₆H₁₀O₃ [M+H]⁺ 299.0926. Found 299.0921.

4.4. Benzo[c]isoxazol-3-ylmethylene-phenyl-amine (11)

2,1-Benzisoxazol-3-carbalddehyde (10) (0.08 g, 0.543 mmol), prepared according to the literature [16], was added to a vigorously stirring solution of aniline (0.051 g, 0.547 mmol) and anhydrous MgSO₄ (0.262 g, 2.175 mmol) in dry toluene (1 mL) under nitrogen atmosphere at 0–5 °C. The reaction mixture was stirred for an additional 15 min at 0–5 °C. The heterogeneous mixture was stirred vigorously for 4 h at room temperature, at which point it cleared and TLC analysis indicated complete consumption of the reactants. The mixture was filtered and the filtrate was evaporated in a vacuum to afford a yellow oil. This was further purified by column chromatography using ethyl acetate-petroleum ether (3:97). Yellow oil, yield: 63%. ¹H NMR (300 MHz, DMSO-d₆): δ: 9.19 (s, 1H, HCN=), 8.20 (d, J = 8.7 Hz, 1H), 7.80 (d, J = 9 Hz, 1H), 7.53 (m, 5H), 7.36 (m, 2H). HRMS (DCI, CH₃CN) m/z calcd for C₁₄H₁₁N₂O [M+H]⁺ 269.0926. Found 269.0921.

4.5. N-Benzo[c]isoxazol-3-yl-N-benzoyl-benzamide (12)

Et₃N (0.045 g, 0.447 mmol) was added to 3-amino-2,1-benzisoxazole (m = 0.060 g, 0.447 mmol) in dry toluene (6 mL), and stirred at 0–5 °C for 10 min and then benzoyl chloride (0.069 g, 0.492 mmol) was added drop-wise. The reaction mixture was then stirred at 0–5 °C for 2 h. The mixture was treated with 5% aqueous HCl (1 × 10 mL) and then extracted with ethyl acetate (3 × 25 mL), and the organic phase was filtered through anhydrous MgSO₄ and vacuum evaporated to afford the crude product. This was further purified by column chromatography using toluene-acetone (v/v = 5/95). Yellow powder, yield: 22%, mp: 105.4 °C. IR (KBr, cm⁻¹): 1683, 1630, 1447, 1190. ¹H NMR (300 MHz, DMSO-d₆): δ: 8.01 (m, 3H), 7.78 (m, 3H), 7.91 (m, 5H), 7.51 (d, J = 6 Hz, 1H), 7.44 (m, 2H). HRMS (DCI, CH₃CN) m/z calcd for C₂₁H₁₅N₂O₃ [M+H]⁺ 343.1083. Found 343.1097.

4.6. Methodology for in vitro biological evaluation

4.6.1. In vitro antiplasmodial and cytotoxicity assays

The in vitro antiplasmodial assay was carried out against the chloroquine-resistant strain (Fcb1) of Plasmodium falciparum and the in vitro cytotoxicity was determined on human breast cancer cells (MCF7) [Cachet et al., 2009; Ribaut et al., 2008]. All these assays were performed as previously reported (Nepveu et al., 2010; Najahi et al., 2013) and details are given in the supplementary data.

4.6.2. Antimicrobial assays

All microorganisms were grown in broth medium with continuous shaking (200 rpm). Bacillus subtilis (CIP 5262), Staphylococcus aureus (CIP 53156) were used as Gram-positive strains. The Gram-negative strains used were Pseudomonas aeruginosa (CIP 82118), Cronobacter sakazakii (CIP 103183). Fungal strains used were Candida albicans (CIP 4872) and Geotrichum candidum (ATCC 204307). The minimal inhibitory concentrations (MICs) were performed as previously reported (Najahi et al., 2013) and details are given in the supplementary data.

Acknowledgement

This work was supported by the European Commission (FP6-LSH-20044-2,3,0-7, STREP no. 018602, Redox antimalarial Drug Discovery, READ-UP). We thank J.-P. Nallet, S. Petit and IDEALP-PHARMA for their scientific contributions.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.arabjc.2013.09.011.

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