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ORIGINAL ARTICLE

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



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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_{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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Benzisoxazoles (Boduszek et al., 1997; Sulikowski and Makosza, 2009; Shelke et al., 2009; Chauhan and Fletcher, 2012) are a promising class of heterocyclics which possess significant pharmacological and biological activities such as anticonvulsant (Stiff and Zemaitis, 1990), antimicrobial (Priya et al., 2005), antipsychotic (Hrib et al., 2003), anticancer (Jain and Kwon, 2003), and affinity for serotonergic and dopaminergic receptors (Nuhlich et al., 1996). In particular, 3-(*N*-benzylpiperidinylolethyl)-1,2-benzisoxazoles inhibit acetyl cholinesterase, making them suitable candidates for the palliative treatment of Alzheimer's disease (Villalobos et al., 1994). 2,1-Benzisoxazoles (anthranils) are fused heteroaromatic systems applied as versatile synthons in fine organic synthesis. This is due to the easy ring opening at the labile *N*-O bond and the ability of 2,1-benzisoxazoles to transform into other

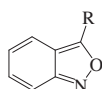


Figure 1 Structure of 3-substituted-2,1-benzisoxazoles **1–13**.

Table 1 Structure, antiplasmodial and cytotoxic activities of 2,1-benzisoxazoles 1–12 .							
Compd.	Structure	M (g mol ⁻¹)/Log <i>P</i> _{calc} ^a	IC ₅₀ (μmol L ⁻¹) FcB1	CC ₅₀ (μmol L ⁻¹) ^b MCF7	Selectivity index ^c MCF-7/FcB1		
1		163.13/1.49	61.3	> 613	> 10		
2		134.13/1.54	70.1	> 480.5	> 10		
3		223.22/3.25	15.0	103	6.9		
4		257.67/3.75	12.8	50.5	3.9		
5		253.25/3.12	12.4	63.2	5.1		
6		237.25/3.35	13.9	63	4.5		
7		238.24/3.11	55.0	25.2	0.5		
8		218.25/2.28	130.6	155.8	1.2		
9		268.26/3.08	69.9	37.3	0.53		
10		147.13/1.36	68.0	> 680	> 10		
11		222.25/3.69	16.4	> 450	> 27.4		
12		342.35/3.59	> 29.2	15.8	< 0.54		
	Chloroquine	319/5.28	0.2	19.4	96		
	Sodium artesunate	406/2.29	0.006	9.8	1633		

^a Log *P*_{calc}: calculated with VCCLAB (<http://www.virtuallaboratory.org/lab/alogps/start.html>).

^b The drug concentration needed to cause a 50% decrease in cell viability; The IC₅₀ SD were always lower than 20% and were discarded for maximum lisibility.

^c Cytotoxicity/antiplasmodial activity ratio.

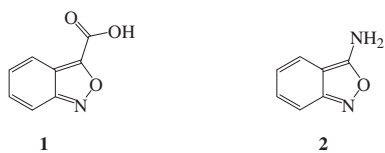


Figure 2 Structure of 2,1-benzisoxazole-3-carboxylic acid (1) and 3-amino-2,1-benzisoxazole (2).

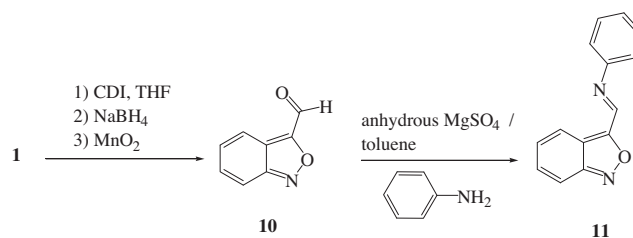
heterocyclic systems, for instance, into acridinones (Ogata et al., 1969). The acridinones are used extensively in the production of dyes and pharmaceuticals (Ogata et al., 1969), and also as biologically active compounds acting as anti-inflammatory agents (Walsh, 1987) and glucosylase inhibitors (Farr 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 antiparasitodal 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 Schröder, 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



Scheme 2 Synthesis of the 2,1-benzisoxazole 11.

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).

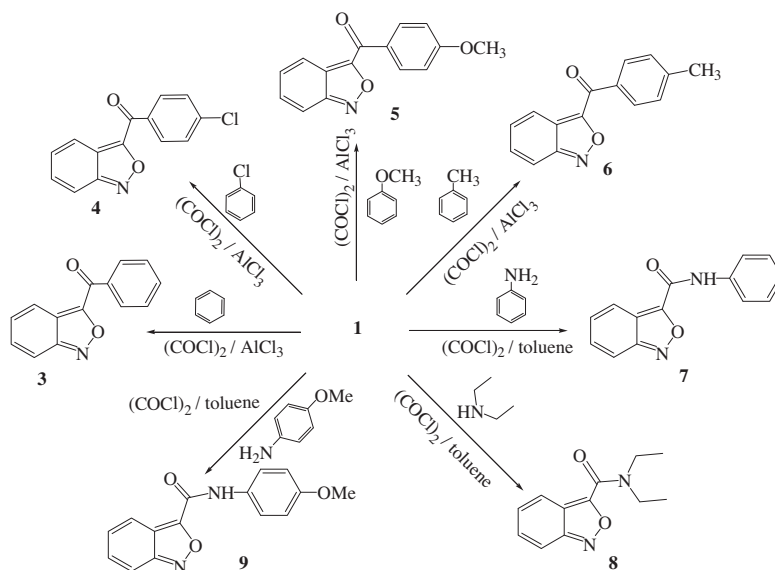
The 3-amino-2,1-benzisoxazole 2 condensed with benzyl chloride in a solution of toluene and triethylamine gave the anthranil 12 at a 22% yield. Approximately 7% of the compound 2 was converted to the 2,1-benzisoxazole 13 as determined by ¹H NMR of the crude material (Scheme 3).

The lipophilicity of the synthesized derivatives 1–12 was expressed in terms of their Log *P*_{calc} values that were calculated with the VCCLAB software. As shown in Table 1, higher Log *P*_{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.

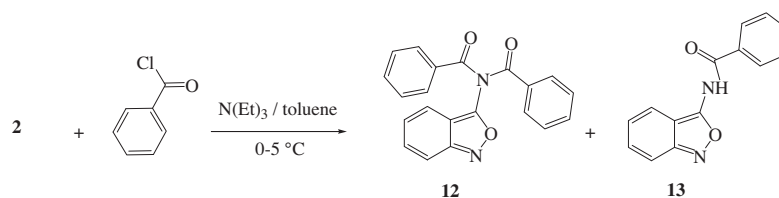
2.2. Pharmacology

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



Scheme 1 Synthesis of 3-substituted-2,1-benzisoxazoles 3–9 starting from the 2,1-benzisoxazole 1.



Scheme 3 Synthesis of 3-substituted-2,1-benzisoxazoles **12–13** starting from the 2,1-benzisoxazole **2**.

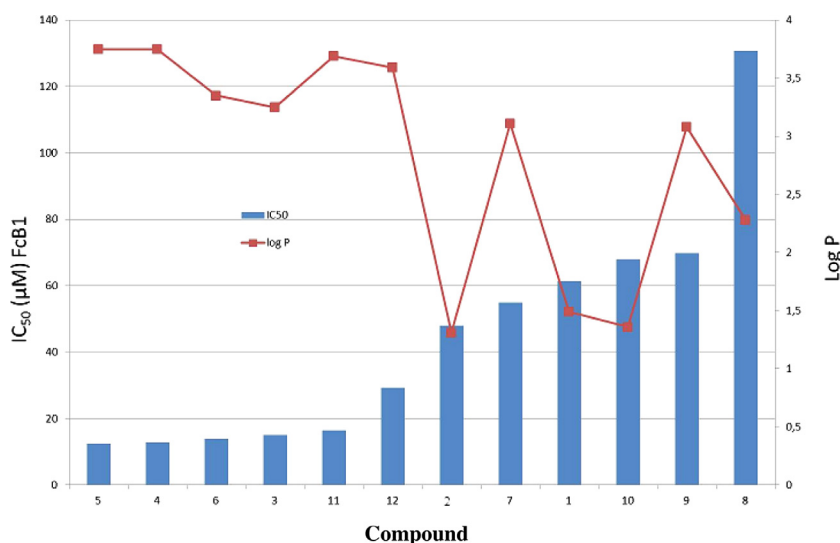


Figure 3 Relationships between $P.f.$ IC_{50} values (blue) and $\log P_{calc}$ in the series (red).

antiplasmodial activities of the 2,1-benzisoxazoles **1–12** expressed as the IC_{50} values, ranged between 12.4 and $130.6\ \mu\text{mol L}^{-1}$ (Table 1). The 2,1-benzisoxazole-3-carboxylic acid **1** where $R^3 = \text{COOH}$ had the weakest activities with IC_{50} values higher than $60\ \mu\text{mol L}^{-1}$. Groups with higher lipophilicity ($\log P > 3$) at position R^3 (acyl/amide/imine) improved the antiplasmodial activity ($IC_{50}/\mu\text{mol L}^{-1} \approx 12\text{--}16$) (**3–6, 11**) while groups (aldehyde) at R^3 with lower lipophilicity ($\log P < 3$) led to a significant decrease in the activity ($IC_{50}/\mu\text{mol L}^{-1}$: **10** = 68). Fig. 3 illustrates the inverse relation between $\log P_{calc}$ and the IC_{50} values.

Cytotoxicity was assayed on the mammalian MCF7 cell line and was higher than $15\ \mu\text{mol L}^{-1}$ with CC_{50} values ranging from 15.8 to $680\ \mu\text{mol L}^{-1}$ (Table 1).

2.2.2. In vitro evaluation of antimicrobial activity

The synthesized 2,1-benzisoxazoles **1–12** were evaluated for their *in vitro* antibacterial activity against representative Gram-positive (*Bacillus subtilis*, *Staphylococcus aureus*) and Gram-negative bacteria (*Pseudomonas aeruginosa*, *Cronobacter sakazakii*) and against two fungi (*Geotrichum candidum*, *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 2 Antimicrobial activity of 2,1-benzisoxazoles.

Compound	<i>Pseudomonas aeruginosa</i>	<i>Cronobacter sakazakii</i>	<i>Bacillus subtilis</i>	<i>Staphylococcus aureus</i>	<i>Geotrichum candidum</i>	<i>Candida albicans</i>
	MIC ^a	MIC	MIC	MIC	MIC	MIC
3	b	b	b	b	44.8	b
7	839.5	b	b	b	b	b
12	584.2	b	b	b	b	b
Tetracycline	56.5	17.9	71.2	2.3	b	b
Ampicillin	71.9	14.3	14.5	0.6	b	b
Amphotericin ^c	b	b	b	b	6.8	0.3

^a MICs: minimum inhibitory concentration ($\mu\text{mol L}^{-1}$).

^b Inactive.

^c Ref. (Sharma et al., 2011).

(MIC = 44.8 $\mu\text{mol L}^{-1}$) against *G. candidum*, and for compounds **7** and **12** (MIC = 839.5 and 584.2 $\mu\text{mol L}^{-1}$, respectively).

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 μmolar 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 10³. 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 (δ_{H} 2.5) or (CD₃)₂SO (δ_{C} 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 Electrothermal 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 \times 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. Benzo[*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-*d*₆) 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-*d*₆) δ : 181.8 (C=O), 157.3 (C), 154.3 (C), 136.3 (C), 134.4 (CH), 132.5 (CH), 130.2 (2CH), 129.6 (CH), 129.4 (2CH), 121.4 (CH), 121 (C), 116.2 (CH). HRMS (DCI, CH₄) m/z calcd for C₁₄H₁₀NO₂ [M + H]⁺ 224.0712. Found 224.0721.

4.2.2. Benzo[*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-*d*₆) 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-*d*₆) δ_{C} 180 (C=O), 156.7 (C), 153.3 (C), 138.9 (C), 134.4 (C), 132.1 (CH), 131.6 (2CH), 129.2 (CH), 129 (2CH), 120.9 (CH), 120.6 (C), 115.7 (CH). HRMS (DCI, CH₄) m/z calcd for C₁₄H₉ClNO₂ [M + H]⁺ 258.0322. Found 258.0328.

4.2.3. Benzo[*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-*d*₆) 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, CH₄) m/z calcd for C₁₅H₁₂NO₃ [M + H]⁺ 254.0817. Found 254.0826.

4.2.4. Benzo[*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-*d*₆) 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, CH₄) m/z calcd for C₁₅H₁₂NO₂ [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 (2 mL) was added drop-wise and the reaction mixture was stirred at room temperature for 1 h. The mixture was treated with aqueous HCl (1 M) and then extracted with ethyl acetate (3 \times 100 mL), and the combined organic phase quenched with NaOH (1 M). Subsequently, the organic phase

was washed with brine (1 × 100 mL), dried over anhydrous MgSO₄, and evaporated in vacuum to afford the crude product. This was further purified by column chromatography using ethyl acetate-petroleum ether (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-*d*₆) 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-*d*₆) δ: 157.5 (C=O), 157.2 (C), 154.9 (C), 138.3 (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, CH₄) *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): 1672, 1612, 1151. ¹H NMR (300 MHz, DMSO-*d*₆) 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, CH₄) *m/z* calcd for C₁₂H₁₆N₂O₂ [M + H]⁺ 219.1134. Found 219.1121.

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

Yellow powder, yield: 27%, mp: 172–174 °C. IR (KBr, cm⁻¹): 3335, 1672, 1629, 1206, 1189. ¹H NMR (300 MHz, DMSO-*d*₆) 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 (dd, *J* = 8.7 Hz, 6.6 Hz, 1H), 6.97 (d, *J* = 9 Hz, 2H), 3.76 (s, 3H). ¹³C NMR (75 MHz, DMSO-*d*₆) 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, CH₄) *m/z* calcd for C₁₅H₁₃N₂O₃ [M + H]⁺ 269.0926. Found 269.0921.

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

2,1-Benzisoxazole-3-carbaldehyde (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 a 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-*d*₆) δ: 9.19 (s, 1H, HC=N), 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, CH₄) *m/z* calcd for C₁₄H₁₁N₂O [M + H]⁺ 223.0871. Found 223.0887.

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 combined organic phase quenched with aqueous NaHCO₃ (1 × 25 mL). The organic phase was subsequently washed with brine (1 × 25 mL), dried over anhydrous MgSO₄, and vacuum evaporated to afford the crude product. This was further purified by column chromatography using toluene-acetone (v/v = 80/20). Yellow powder, yield: 22%, mp: 105.4 °C. IR (KBr, cm⁻¹): 1683, 1630, 1447, 1190. ¹H NMR (300 MHz, DMSO-*d*₆) 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, CH₄) *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* antipalmodial and cytotoxicity assays

The *in vitro* antipalmodial 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.

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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.arabj.2013.09.011>.

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