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Short communication

Synthesis and antiprotozoal activity of some 2-(trifluoromethyl)-1*H*-benzimidazole bioisosteres

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Abstract

A series of 2-(trifluoromethyl)-1*H*-benzimidazole derivatives with various 5- and 6-position bioisosteric substituents (–Cl, –F, $–CF_3$, –CN), namely **1**–**7**, were prepared using a short synthetic route. Each analogue was tested in vitro against the protozoa *Giardia intestinalis* and *Trichomonas vaginalis* in comparison with albendazole and metronidazole. Several analogues had IC_{50} values < 1 μM against both species, which make them significantly more potent than either standard. Compound **4** [2,5(6)-bis(trifluoromethyl)-1*H*-benzimidazole], was 14 times more active than albendazole against *T. vaginalis*. This compound (**4**) also showed moderate antimalarial activity against W2 and D6 strains of *Plasmodium falciparum* (5.98 and 6.12 μM, respectively). Studying further structure activity relationships through the use of bioisosteric substitution in these benzimidazolic derivatives should provide new leads against protozoal and possibly malarial diseases.

Keywords: Benzimidazoles; *Trichomonas vaginalis*; Bioisosteric replacement

1. Introduction

2-(Trifluoromethyl)benzimidazoles are known as an important class of compounds due to their wide range of biological activity acting as antiviral, antifungal, antibacterial and anticancer drugs [1–4]. More recently, antiparasitic activities of this class of compounds have been reported [5,6], which is consistent with earlier observations concerning the giardicidal activity of albendazole and mebendazole [7,8].

Our studies about the antiparasitic activity of 2-(trifluoromethyl)benzimidazole derivatives have shown high potential as antiprotozoal agents. When compared to metronidazole, the drug of choice [5], 2-(trifluoromethyl)-1*H*-benzimidazole (**1**) and 5(6)-Chloro-2-(trifluoromethyl)-1-*H*-benzimidazole (**2**), showed higher and equal activity, respectively. As part of our search for basic information about the structural requirements for antiparasitic activity, we now report the synthesis and antiprotozoal activity of 2-(trifluoromethyl)benzimidazole derivatives shown in Table 1. Three of them (**3**, **4** and **5**) were obtained by isosteric replacement [9] at position 5(6) of Compound **1.** The replacement included fluorine, trifluoromethyl and cyanide groups (Fig. 1). The two 1-methyl regioisomeres of **4** gave rise to compounds **6** and **7**. The in vitro antiparasitic activity of these compounds on an intestinal protozoan (*Giardia intestinalis),* a urogenital tract parasite (*Trichomonas vaginalis*) and red blood cell parasite (*Plasmodium falciparum*) is also reported in this paper.

2. Chemistry

Compounds **1** and **2** were prepared as described before in [5]. For the synthesis of compounds **3**, **4** and **5**, the sequence

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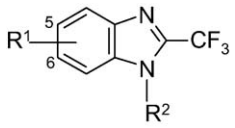
Table 1
Structure of synthesized 2-(trifluoromethyl)-1-*H*-benzimidazoles (**1**–**7**)



Compound	R^1	R^2
1	5(6)-H	H
2	5(6)-Cl	H
3	5(6)-F	H
4	5(6)-CF_3	H
5	5(6)-CN	H
6	5-CF_3	CH_3
7	6-CF_3	CH_3

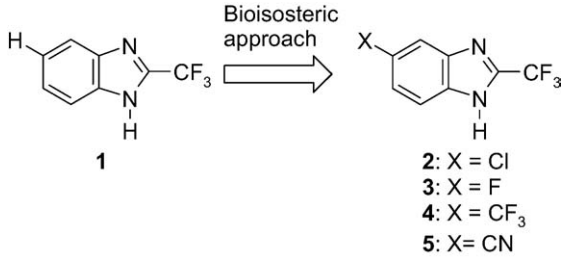
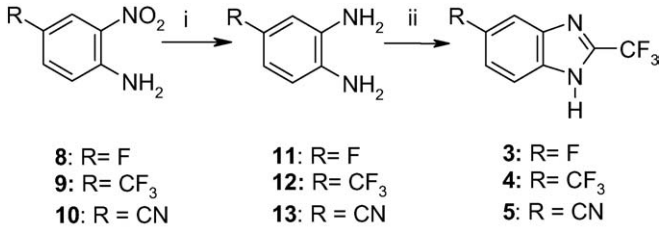


Fig. 1. 2-(Trifluoromethyl)-1*H*-benzimidazole (**1**) and their bioisosteres prepared in this work.

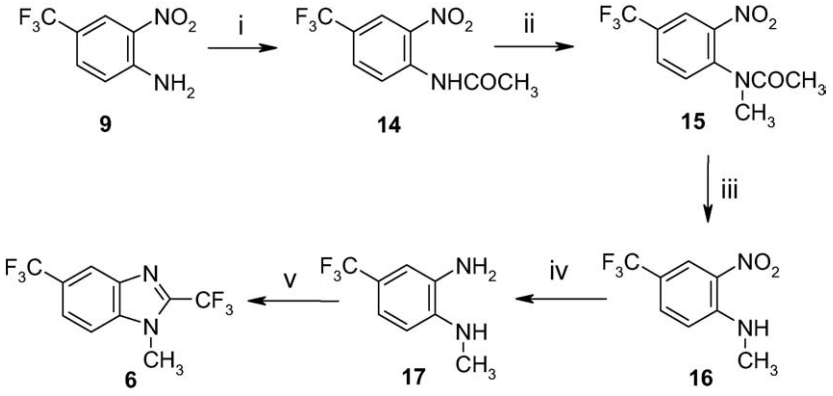
shown in Scheme 1 was followed. Starting from the corresponding 2-nitroanilines **8**, **9** or **10**, through reduction with H_2 and Ni-Raney in ethanol, 1,2-phenylenediamines **11**–**13** were obtained. Reaction of these with CF_3COOH using HCl as catalyst yielded compounds **3**–**5**. Since the 5(6)-(trifluoromethyl)-substituted compound **4** exhibited the best antiprotozoal activity against *T. vaginalis* and was more active than metronidazole, we decided to synthesize its 1-methyl regioisomers **6** and **7**. Compound **6** was prepared following the reactions showed in Scheme 2. Starting from **9**, the acetylation with Ac_2O using H_2SO_4 as catalyst afforded the acetanilide **14**. This was treated with dimethyl sulfate and KOH to give the *N*-methylated acetamide **15**. Hydrolysis of **15** with H_2SO_4 led to the *N*-methyl-2-nitroaniline **16**, which upon catalytic reduction with H_2 and Ni-Raney generated the *o*-phenylenediamine **17**. Finally, boiling **17** with CF_3COOH afforded the benzimidazole derivative **6**. For the synthesis of the regioisomeric derivative **7**, *o*-phenylenediamine **24** was first prepared through the series of reactions shown in Scheme 3. Thus, 3-(trifluoromethyl)aniline **18**, through nitration with KNO_3 in trifluoroacetic anhydride and hydrolysis of the nitration product **19** with H_2SO_4, preferentially yielded the 2-Nitro-5-(trifluoromethyl)aniline **20**. The acetylation of **20** with Ac_2O, and *N*-methylation of acetanilide **21** led to *N*-Methyl-2-nitroacetanilide **22**, which upon acid hydrolysis followed by reduction of **23** with Ni-Raney, afforded the required *o*-phenylenediamine **24**. Cyclocondensation of **24** with CF_3COOH, as shown before, gave **7**. We also prepared compounds **6** and **7** by direct methylation of **4** using methyl iodide, but the separation of the regioisomers was unsuccessful. Solid compounds were purified by recrystallization. The structures of the purified products were established by ^1H NMR, ^{13}C NMR, mass spectrometry, and HRMS data.



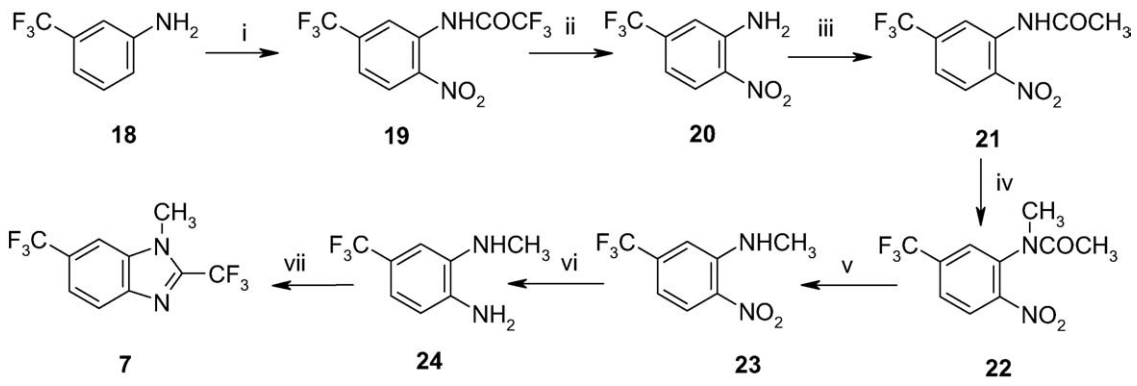
Scheme 1. Reagents: **i)** H_2, Ni-Raney; **ii)** CF_3COOH, HCl, reflux.

3. Biological results and discussion

In this study three new 2-(trifluoromethyl)benzimidazole derivatives (Compounds **3**–**5**) were synthesized and tested in vitro as antiprotozoal agents against *G. intestinalis*, *T. vaginalis* and *P. falciparum*. The main features of these compounds are the isosteric substitution of the hydrogen atom at position 5(6) by trifluoromethyl, fluorine and cyanide groups in order to determine bioisosteric equivalence, enhancement of solubility and absorption, and potential antiprotozoal activity. The 1-methyl regioisomers of Compound **4** were prepared in order to determine the importance of hydrogen at position 1 on the antiprotozoal activity.



Scheme 2. Reagents: **i)** Ac_2O, H_2SO_4 (catalyst); **ii)** monoglyme, KOH, $(CH_3)_2SO_4$; **iii)** H_2SO_4, H_2O; **iv)** H_2, Ni-Raney, MeOH; **v)** CF_3COOH, HCl, reflux.



Scheme 3. Reagents: **i)** TFAA, KNO_3; **ii)** K_2CO_3,EtOH; **iii)** Ac_2O, H_2SO_4 (catalyst); **iv)** monoglyme, KOH, $(CH_3)_2SO_4$; **v)** H_2SO_4, H_2O; **vi)** H_2, Ni-Raney, MeOH; **vii)** CF_3COOH, HCl, reflux.

Biological assay results shown in Table 2, against *G. intestinalis*, indicate that none of the compounds synthesized were more active than albendazole. However, compounds **3**, **4** and **6** showed better activity than metronidazole. Compound **2** was as active as metronidazole. Little or no activity was found for the 1-methyl regioisomer **7**. In contrast, the activity of 1,5 regioisomer **6** was similar to that of metronidazole. Compound **3**, with a fluorine atom at position 5(6) was 2.5 times more active than metronidazole. Fluorine presents the advantage of having a van der Waals radius comparable to that of hydrogen and therefore, can be used to protect the metabolically sensitive 5(6) position of a benzimidazole molecule. When substituting hydrogen with a cyanide group at position 5 of the benzimidazole ring, the activity decreased.

In the assay against *T. vaginalis*, compounds **2**–**5** were more active than albendazole. The same assay showed compound **4**, with CF_3 at position 5(6), to be as active as metronidazole. Interestingly, compound **4** was 14 times more active than albendazole against *T. vaginalis*. Regioisomeric compounds **6** and **7** showed less potency than albendazole and had similar activity against *T. vaginalis*.

The in vitro antimalarial activity exhibited by these compounds was poor when compared to Mefloquine, which IC_{50} values against W2 and D6 strains of *P. falciparum* are approximately 0.048 and 0.028 μM, respectively. Only compounds **2** and **4** (with Cl and CF_3 at position 5(6), respectively) were moderately active against W2 and D6 strains of *P. falciparum*. Compound **4** was the most active, with IC_{50} values of approximately 6 μM against either isolate. This spectrum can be explained because the CF_3 group is comparable in size to chlorine and could advantageously replace it to give a potent bioisoster. A chlorine substituent simultaneously produces an increase in lipophilicity, an electron attracting effect and metabolic obstruction. Thus, bioisosterism represents one approach used by the medicinal chemist for the rationale modification of lead compounds into safer and more clinically relevant agents.

These compounds are fully compatible with Lipinski's rule [10] (Table 3), which should allow for the development of additional antiprotozoal analogues. Their advantages include:

- physical properties known to be compatible with desirable pharmacokinetic (low molecular weight, favorable *C* log *P*, favorable hydrogen bond donating and accepting capabilities);
- potency and efficacy, with IC_{50} values at the low micromolar level;
- simple synthetic access and thus low production costs;
- bioisosteric groups improving the likelihood of reasonable solubility. Further optimization and pharmacokinetics characterization of this series are ongoing.

Table 2
In vitro susceptibility of *G. intestinalis*, *T. vaginalis* and *P. falciparum* to synthesized compounds, metronidazole and albendazole

Compound	*G. intestinalis*	*T. vaginalis*	*P. falciparum*	
			D6	*W2*
	IC_{50} (μM)[a]	IC_{50} (μM)	IC_{50} (μM)	IC_{50} (μM)
Metronidazole	1.226 ± 0.125	0.236 ± 0.016	NT	NT
Albendazole	0.038 ± 0.003	3.390 ± 0.125	> 20	> 20
1	0.107 ± 0.017	3.134 ± 0.130	> 20	> 20
2	1.282 ± 0.120	0.451 ± 0.025	11.70 ± 0.33	12.52 ± 0.43
3	0.489 ± 0.018	3.147 ± 0.120	> 20	> 20
4	0.672 ± 0.020	0.232 ± 0.021	5.98 ± 0.25	6.12 ± 0.32
5	1.791 ± 0.130	2.286 ± 0.095	> 20	> 20
6	1.144 ± 0.135	4.098 ± 0.125	> 20	> 20
7	8.850 ± 0.128	5.320 ± 0.095	> 20	> 20

NT: not tested.
[a] Data are presented as mean ± S.E.M.

Table 3
The antiprotozoal benzimidazoles have physical properties compatible with reasonable pharmacokinetics and drug availability

Compound	mol wt	*C* log *P*	Number of H bond donors	Number of H bond acceptors	Number of criteria met
rule	< 500	< 5	< 5	< 10	at least 3
1	186	2.40	1	1	all
2	220	3.14	1	1	all
3	204	2.60	1	1	all
4	254	2.97	1	1	all
5	211	1.83	1	2	all
6	268	3.28	0	1	all
7	268	3.28	0	1	all

4. Conclusion

Substitution at position 5(6) of compound **1** with fluorine or a trifluoromethyl group led to bioisosters more potent than metronidazole against *G. intestinalis*. However, against *T. vaginalis*, compound **4** was the only one as active as metronidazole. This compound also showed moderate antimalarial activity against W2 and D6 strains of *P. falciparum.* The results obtained with the synthesized analogues as antiprotozoal agents are very promising indeed since they broaden the knowledge of the activity of these versatile benzimidazole derivatives.

5. Experimental

5.1. Instruments

Melting points were determined on a Büchi B-540 melting point apparatus and are uncorrected. Reactions were monitored by TLC on 0.2 mm precoated silica gel 60 F_{254} plates (E. Merck). ^{1}H NMR and ^{13}C NMR spectra were measured with a Varian EM-390 (300 and 75.5 MHz) spectrometer. Chemical shifts are given in ppm relative to tetramethylsilane (Me_4Si, $\delta = 0$) in $CDCl_3$; *J* values are given in Hz. The following abbreviations are used: s, singlet; d, doublet; q, quartet; dd, doublet of doublet; t, triplet; m, multiplet; bs, broad signal. MS were recorded on a JEOL JMS-SX102A spectrometer by electron impact (EI). Catalytic hydrogenations were carried out in a Parr shaker hydrogenation apparatus. Starting materials **8**–**10** and **18** were commercially available (Aldrich). The *C* log *P* values were obtained using ACD/labs software v.4.5.

5.2. General method of synthesis of 2-(trifluoromethyl)-1H-benzimidazoles (*1*–*7*)

The appropriate 1,2-phenylenediamine (0.0313 mol), 1.6 equivalents of CF_3COOH and one drop of concentrated HCl were heated under reflux in a N_2 atmosphere for 3–4 h. TLC was used to monitor the reaction. The cooled mixture was neutralized with saturated $NaHCO_3$ solution, and the crude benzimidazole was extracted with AcOEt. The solvent was removed under vacuum, and the resulting solid was isolated by filtration through a fritted 60 ml glass funnel packed with Al_2O_3, neutral type. Applying this technique the following compounds were prepared:

5.2.1. 2-(Trifluoromethyl)-1H-benzimidazole (*1*)

Eluted with hexane and recrystallized from ethanol–water. Yield 4.7 g (81%) of white solid. M.p. 208–210 °C (Lit. 209–211 °C [5]). ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.41 (m, 2H, H-5, H-6), 7.75 (m, 2H, H-4, H-7), 8.21 (bs, 1H, NH) ppm; ^{13}C NMR (75.5 MHz, DMSO-d_6) δ 116.8 (C-4, C-7), 119.1 (q, CF_3, $J = 285.6$ Hz), 124.8 (C-5, C-6), 138.3 (C-7a), 138.5 (C-3a), 141.1 (q, C-2, $J = 39.1$ Hz) ppm; MS: *m/z* (% relative intensity) 186 (M^+, 100), 166 (80); HRMS: calc. for $C_8H_5F_3N_2$: 186.0404, found: 186.0410.

5.2.2. 5(6)-Chloro-2-(trifluoromethyl)-1H-benzimidazole (*2*)

Eluted with hexane and recrystallized from ethanol. Yield 5.5 g (68%) of white solid. M.p. 196–198 °C (Lit. 197–199 °C [5]). ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.63 (dd, 1H, H-6, $J_{6,7} = 8.7$, $J_{6,4} = 2.2$ Hz), 7.68 (d, 1H, H-7, $J_{7,6} = 8.7$ Hz), 7.99 (d, 1H, H-4, $J_{4,6} = 2.2$ Hz) 13.4 (bs, 1H, NH) ppm; ^{13}C NMR (75.5 MHz, DMSO-d_6) δ 116.6 (C-7), 119.2 (q, CF_3, $J = 285.6$ Hz), 118.33 (C-4), 125.78 (C-6), 128.3 (C-5), 136.9 (C-7a), 137.4 (C-3a), 143.3 (q, C-2, $J = 39.2$ Hz) ppm; MS: *m/z* (% relative intensity) 222 (M^{+2}, 30), 220 (M^+, 100), 200 (50); HRMS: calc. for $C_8H_4ClF_3N_2$: 220.0015, found: 220.0028.

5.2.3. 5(6)-Fluoro-2-(trifluoromethyl)-1H-benzimidazole (*3*)

Eluted with hexane and recrystallized from cyclohexane-CH_2Cl_2. Yield 7.98 g (93%) of white solid. M.p. 220–221 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.19 (dd, 1H, H-6, $J_{6,7} = 10.0$, $J_{6,4} = 1.0$ Hz), 8.03 (d, 1H, H-4, $J_{4,6} = 1.0$ Hz), 7.87 (d, 1H, H-7, $J_{7,6} = 10.0$ Hz), 12.9 (bs NH) ppm; ^{13}C NMR (75.5 MHz, DMSO-d_6) δ119.0 (q, CF_3, $J = 271.6$ Hz), 111.5 (d, C-4, $J = 21.1$ Hz), 115.1 (d, C-6, $J = 21.1$ Hz), 115.5 (d, C-7, $J = 7.8$ Hz), 143.3 (q, C-2, $J = 39.1$ Hz), 155.8 (d, C-5, $J = 245.1$ Hz) ppm; MS: *m/z* (% relative intensity) 204 (M^+, 100), 184 (74); HRMS: calc. for $C_8H_4F_4N_2$: 204.1241, found: 204.1239.

5.2.4. 2,5(6)-Bis(trifluoromethyl)-1H-benzimidazole (*4*)

Eluted with $CHCl_3$ and recrystallized from ethanol. Yield 1.7 g (68%) of white solid. M.p. 201–202 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.66 (dd, 1H, H-6, $J_{6,4} = 1.3$, $J_{6,7} = 8.7$ Hz), 7.89 (d, 1H, H-7, $J_{7,6} = 8.7$ Hz), 8.11 (d, 1H, H-4, $J_{4,6} = 1.3$ Hz), 13.98 (bs, 1H, N-H) ppm; ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 115.43 (C-4), 116.92 (C-7), 118.72 (q,

CF_3-C2, $J = 271.8$ Hz), 120.85 (C-6), 124.61 (q, CF_3-C5, $J = 271.8$ Hz), 124.62 (q, C-5, $J = 32.2$ Hz), 138.32 (C-3a), 139.06 (C-7a), 142.61 (q, C-2, $J = 40.3$ Hz) ppm; MS: *m/z* (% relative intensity) 254 (M^+, 100), 234 (60), 215 (22), 204 (5), 184 (15); HRMS: calc. for $C_9H_4F_6N_2$: 254.0278, found: 254.0278.

*5.2.5. 5(6)-Cyano-2-(trifluoromethyl)-1H-benzimidazole (**5**)*

Eluted with hexane and recrystallized from cyclohexane-CH_2Cl_2. Yield 7.98 g (93%) of white solid. M.p. 183–184 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.48 (dd, 1H, H-6, $J_{6,7} = 9.0$, $J_{6,4} = 0.9$ Hz), 7.86 (d, 1H, H-7, $J_{7,6} = 9.0$ Hz), 8.34 (d, 1H, H-4, $J_{4,6} = 0.9$ Hz), 14.4 (bs, 1H, N-H) ppm; ^{13}C NMR (75.5 MHz, DMSO-d_6) δ 106.3 (C-5), 118.6 (q, CF_3, $J = 271.8$ Hz), 117 (C-7), 119.2 (CN), 126.7 (C-4), 127.3 (C-6), 142.9 (q, C-2, $J = 39.1$ Hz); MS: *m/z* (% relative intensity) 211 (M^+, 100), 191 (68); HRMS: calc. for $C_9H_4F_3N_3$: 211.1436, found: 211.1438.

*5.2.6. 1-Methyl-2,5-bis(trifluoromethyl)-1H-benzimidazole (**6**)*

Eluted with hexane and recrystallized from ethanol. Yield 10 g (71%) of white solid. M.p. 56–58 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.00 (s, 3H, CH_3), 7.55 (d, 1H, H-7, $J_{7,6} = 8.7$ Hz), 7.69 (dd, 1H, H-6, $J_{6,4} = 1.5$, $J_{6,7} = 8.7$ Hz), 8.16 (d, 1H, H-4, $J_{4,6} = 1.5$ Hz) ppm; ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 31.12 (d, *N*-CH_3, $J = 2.0$ Hz), 110.83 (C-7), 118.74 (q, CF_3-C2, $J = 271.9$ Hz), 119.50 (q, C-4, $J = 4.0$ Hz), 122.14 (q, C-6, $J = 4.1$ Hz), 124.34 (q, CF_3-C5, $J = 275.9$ Hz), 126.39 (q, C-5, $J = 33.2$ Hz), 137.83 (C-3a), 140.38 (C-7a), 142.77 (q, C-2, $J = 38.3$ Hz) ppm; MS: *m/z* (% relative intensity) 268 (M^+, 100), 249 (30), 218 (10), 197 (5); HRMS: calc. for $C_{10}H_6F_6N_2$ (M^+) *m/z*: 268.0435, found 268.0440.

5.2.7. 1-Methyl-2,6-bis(trifluoromethyl)-1H-benzimidazole (7)

Eluted with hexane and recrystallized from ethanol. Yield 2.4 g (64%) of white solid. M.p. 122–124 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.05 (s, 3H, CH_3), 7.66 (dd, 1H, *H*-5, $J_{5,7} = 1.7$, $J_{5,4} = 8.7$ Hz), 8.01 (d, 1H, *H*-4, $J_{4,5} = 8.7$ Hz), 8.31 (d, 1H, *H*-7, $J_{7,5} = 1.7$ Hz) ppm; ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 31.44 (d, *N*-CH_3, $J = 2.1$ Hz), 110.36 (q, C-7, $J = 4.9$ Hz), 118.71 (q, CF_3-C2, $J = 272.2$ Hz), 119.89 (q, C-5, $J = 3.5$ Hz), 122.82 (C-4), 124.57 (q, CF_3-C6, $J = 272.9$ Hz), 125.61 (q, C-6, $J = 31.7$ Hz), 126.38 (C-7a), 135.68 (C-3a), 142.65 (q, C-2, $J = 25.4$ Hz) ppm; MS: *m/z* (% relative intensity) 268 (M^+, 100), 249 (30), 218 (20), 197 (20), 145 (10); HRMS: calc. for $C_{10}H_6F_6N_2$ (M^+) *m/z*: 268.0435, found 268.0431.

*5.3. General method of synthesis of 1,2-phenylenediamines **11**–**13, 17** and **24***

A mixture of adequate substituted 2-nitroaniline (6.37 g, 0.0282 mol), EtOH (100 ml) and 10% Ni-Raney (300 mg) was hydrogenated at 25 °C until cessation of H_2 uptake. The catalyst was filtered off on a Whatman paper number 2, washed with EtOH, and the filtrate concentrated to provide a dark purple–colored liquid, which was used immediately in a subsequent step without purification.

*5.4. Synthesis of precursors **14**–**16, 19**–**23***

*5.4.1. N-[2-Nitro-4-(trifluoromethyl)phenyl]acetamide (**14**)*

A stirred mixture of 4-(trifluoromethyl)-2-nitroaniline (12.2 g, 0.0591 mol), acetic anhydride (9.78 g, 9.06 ml, 0.0882 mol, 1.5 eq) and three drops of H_2SO_4 was heated at 80 °C for 1 h. The mixture was cooled, worked up by addition of cold water and filtered by suction. The crude product was recrystallized from hexane. Yield 14.2 g (97%) of white crystals. M.p. 112–113 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.38 (s, 3H, CO-CH3), 7.87 (dd, 1H, H-5, $J_{5,3} = 3.1$, $J_{5,6} = 9.0$ Hz), 8.51 (d, 1H, H-3, $J_{3,5} = 3.1$ Hz), 9.06 (d, 1H, H-6, $J_{6,5} = 9.0$ Hz) ppm; MS: m/z (% relative intensity) 248 (M+,10), 206 (100), 176 (20).

*5.4.2. N-methyl-N-[2-nitro-4-(trifluoromethyl)phenyl] acetamide (**15**)*

Into a stirred mixture of **9** (14.1 g, 0.0572 mol) in dimethyl sulfate (10.82 g, 8.11 ml, 0.0858 mol, 1.5 eq) and monoglyme (14 ml) was added a solution of KOH 50% m/v (4.81 g, 0.0858 mol, 1.5 eq) at 32–35 °C. The mixture was cooled, worked up by addition of cold water and extracted with EtOAc. The combined organic extracts were washed with brine, dried with anhydrous Na_2SO_4 and concentrated in vacuo to give an orange liquid (14.78 g, 98.6%), which was immediately hydrolyzed in the next step.

*5.4.3. N-methyl-2-nitro-4-(trifluoromethyl)aniline (**16**)*

A solution of **15** (14 g, 0.0533 mol) in concentrated sulfuric acid (15 ml) and water (1 ml) was heated at 80–90 °C for 15 min, and then cooled to room temperature. Ice (250 g) was added and the precipitated solid was removed by filtration and washed several times with water until neutral pH. It was recrystallized from ethanol to give yellow pale needles (11.72 g, 98%). M.p. 73–75 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.05 (s, 3H, *N*-CH_3), 6.94 (d, 1H, H-6, $J_{6,5} = 8.8$ Hz), 7.65 (dd, 1H, H-5, $J_{5,6} = 8.8$, $J_{5,3} = 1.8$ Hz), 8.48 (d, 1H, H-3, $J_{3,5} = 1.8$ Hz) ppm; MS: *m/z* (% relative intensity) 220 (M^+,60), 201 (10), 145 (30), 127 (30), 105 (100).

*5.4.4. 2,2,2-Trifluoro-N-[2-nitro-5-(trifluoromethyl)phenyl] acetamide (**19**)*

Into a cooled solution of compound **18** (6 g, 0.0372 mol) in trifluoroacetic anhydride (10 ml) was added potassium nitrate (3.75 g, 0.0372 mol, 1 eq) and the resulting solution was stirred at 0 °C for 3 h at 25 °C, then, all volatiles were removed in vacuo, and the solid residue was purified by flash chromatography eluted with petroleum ether to give a single yellow product (3.7 g, 33%). M.p. 87–89 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 11.47 (sa, 1H, *N*-H), 7.78 (dd, 1H, H-4, $J_{4,6} = 1.9$, $J_{4,3} = 9.2$ Hz), 8.31 (d, 1H, H-3, $J_{3,4} = 9.2$ Hz), 9.00 (d, 1H, H-6, $J_{6,4} = 1.9$ Hz) ppm; MS: *m/z* (% relative intensity) 302 (M^+, 10), 283 (10), 233 (100), 205 (80).

*5.4.5. 2-Nitro-5-(trifluoromethyl)aniline (**20**)*

Aqueous potassium carbonate solution (1 M, 15 ml) was added to the trifluoroacetamide **19** (3.7 g, 0.0124 mol) in ethanol (15 ml). The solution was heated at 50 °C for 3 h. The mixture was cooled, worked up by addition of cold water, filtered by suction and recrystallized from ethanol to give orange crystals (2.45 g, 96%). M.p. 105–106 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.04 (sa, 2H, *N*-H_2), 7.57 (d, 1H, H-6, $J_{6,4}$ = 1.9 Hz), 7.61 (dd, 1H, H-4, $J_{4,6}$ = 1.9, $J_{4,3}$ = 9.0 Hz), 7.91 (d, 1H, H-3, $J_{3,4}$ = 9.0 Hz) ppm; MS: *m/z* (% relative intensity) 206 (M^+, 100), 190, (15), 187 (20), 168 (5), 160 (30),137 (23).

*5.4.6. N-[2-Nitro-5-(trifluoromethyl)phenyl]acetamide (**21**)*

A stirred mixture of 2-nitro-5-(trifluoromethyl)aniline (2.45 g, 0.0118 mol), acetic anhydride (1.81 g, 1.67 ml, 0.0178 mol, 1.5 eq) and three drops of H_2SO_4 was heated at 80 °C for 1 h. The mixture was cooled, worked up by addition of cold water, filtered by suction and the crude product recrystallized from hexane. Yield 2.84 g (97%) of white crystals. M. p. 125–127 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.00 (s, 3H, CO-CH_3), 7.86 (d, 1H, H-6, $J_{6,4}$ = 1.9 Hz), 8.14 (dd, 1H, H-4, $J_{4,6}$ = 1.9, $J_{4,3}$ = 9.0 Hz), 9.36 (d, 1H, H-3, $J_{3,4}$ = 9.0 Hz) ppm; MS: *m/z* (% relative intensity) 248 (M^+,10), 206 (100), 176 (30).

*5.4.7. N-methyl-N-[2-nitro-5-(trifluoromethyl)phenyl] acetamide (**22**)*

Into a stirred mixture of **21** (2.48 g, 0.0100 mol), in dimethyl sulfate (1.89 g, 1.42 ml, 0.0150 mol, 1.5 eq) and monoglyme (10 ml), was added a solution of KOH 50% m/v (0.841 g, 0.0150 mol, 1.5 eq) at 32–35 °C. The mixture was cooled, worked up by addition of cold water and extracted with EtOAc. The combined organic extracts were washed with brine, dried with anhydrous Na_2SO_4 and concentrated in vacuo to give an orange liquid (2.58 g, 98%), which was immediately hydrolyzed in the next step.

*5.4.8. N-methyl-2-nitro-5-(trifluoromethyl)aniline (**23**)*

Water (1 ml) was added to a solution of **22** (2.58 g, 0.098 mol) in concentrated sulfuric acid (5 ml). The solution was heated at 80–90 °C for 15 min, and then cooled to room temperature. Ice (250 g) was added and the orange–yellow precipitated solid was removed by filtration and washed several times with water until neutral pH. It was recrystallized from ethanol to give orange crystals (2.2 g, 99%). M.p. 86–88 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.07 (s, 3H, *N*-CH_3), 7.40 (d, 1H, H-6, $J_{6,4}$ = 2.0 Hz), 7.62 (dd, 1H, H-4, $J_{4,6}$ = 2.0, $J_{4,3}$ = 9.0 Hz), 7.93 (d, 1H, H-3, $J_{3,4}$ = 9.0 Hz) ppm; MS: *m/z* (% relative intensity) 220 (M^+,70), 201 (15), 145 (30), 127 (50), 105 (100).

5.5. Biological assays

5.5.1. In vitro antiprotozoal assay

G. intestinalis strain IMSS:0989:1 and *T. vaginalis* strain GT3 were cultured in TYI-S-33 modified medium, supplemented with 10% calf serum and bovine bile. In vitro susceptibility assays were performed using a method previously described in [7]. Briefly: 4×10^4 trophozoites of *G. intestinalis* or *T. vaginalis* were incubated for 48 h at 37 °C with increasing concentrations of synthesized compounds, albendazole, and metronidazole. As the negative control, trophozoites were incubated with dimethylsulphoxide (DMSO) used in the experiments. After the incubation, an aliquot (5.0 μl) of the treated trophozoites were subcultured for another 48 h in fresh medium alone. At the end of this period, trophozoites were counted and the 50% inhibitory concentration (IC_{50}) was calculated by Probit analysis. Experiments were carried out in triplicate and repeated at least twice.

5.5.2. In vitro antimalarial assay

The in vitro potency of each of the analogues was tested using the tritiated hypoxanthine method as described by Milhous et al. [11] with minor modifications. The parent analogues were dissolved in DMSO as 50.0 mg/ml stock solutions. The stock solutions were diluted 1000-fold in folate-free media (RPMI-1640) to get the 50.0 or 1.0 μg/ml starting concentrations, respectively. Twofold dilutions of the starting concentration were made in folate-free media and 20 μl per well was added to a 96-well culture plate. A 0.5% parasite concentration was diluted fourfold in folate-free media and 180 μl per well was added to each culture plate. Five 10-fold serial dilutions were made to evaluate a range from 5000 ng/ml to 5 pg/ml for the compounds. The plates were maintained at 37 °C for 48 h, then, 25 μl of [^{3}H]-hypoxanthine (15 μCi/ml) were added, and the parasite plates were incubated for an additional 24 h. At 72 h, parasites were harvested onto Unifilter-96 microplates. The filter plates were air-dried and 50 μl per well scintillation fluid were added. Radioactive emissions were counted in a TopCount NXT (Perkin–Elmer, Wellesley, MA). Each analogue was assayed against each parasite strain in triplicate on three separate occasions. Parasite growth inhibition was measured by the decreased accumulation of [^{3}H]-hypoxanthine used in the purine nucleotide salvage pathway. The IC_{50} value was determined by sigmoid dose-response non-linear regression analysis. Two well characterized *P. falciparum* clones W2 (Indochina III/CDC) and D6 (Sierra Leone I/CDC), representing antifolate-resistant and -sensitive strains, respectively, were assayed [12,13].

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