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CONCISE ARTICLE

Synthesis of carboxyimidamide-substituted benzo[*c*][1,2,5]oxadiazoles and their analogs, and evaluation of biological activity against *Leishmania donovani*

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A facile synthesis route to carboxyimidamide-substituted benzoxadiazoles and related derivatives was developed. A total of 25 derivatives were synthesized. They were evaluated for antileishmanial activity by inhibition of *Leishmania donovani* axenic amastigote growth using a fluorescent viability microplate assay. The most promising derivative (**14**) demonstrated an antileishmanial EC_{50} of 4.0 µM, and it also showed activity in infected macrophages (EC_{50} 5.92 µM) without signs of cytotoxicity.

1. Introduction

Leishmaniasis is a spectrum of human diseases caused by at least 20 different species of protozoan parasites. These parasites are responsible for three major diseases: cutaneous, visceral, and mucocutaneous. Over 300 million people are at risk.[1] Visceral leishmaniasis (VL), caused primarily by *L. donovani* (anthroponotic) or *L. infantum/L. chagasi* (zoonotic), is fatal if left untreated. There are an estimated 300 000 cases of VL per year, and 20 000 to 40 000 deaths. Over 90% of new cases occur in six countries: Bangladesh, Brazil, Ethiopia, India, South Sudan, and Sudan. Although two new drugs, miltefosine and paromomycin, are currently in use and combination therapy is becoming more common,[2] an urgent need for new antileishmanial chemotypes persists. The major issues to be confronted in *Leishmania* treatment are the toxicity of primary medicines and the development of parasite resistance. In addition, poor efficacy in the case of visceral leishmaniasis/HIV coinfection has raised the need for new antileishmanial treatments.[3]

In our previous study, 2-arylbenzimidazole and benzo[*c*][1,2,5]oxadiazole derivatives showed activity against the intracellular Gram-negative bacterium *Chlamydia pneumoniae*.[4] In that study, a similarity-based model of *C. pneumoniae* dimethyladenosine transferase was used to virtually screen compounds from commercially available databases against *C. pneumoniae*. The 2-arylbenzimidazoles found were then tested and proven active against *L. donovani*.[5] This prompted us to consider benzo[*c*][1,2,5]oxadiazole-derived compounds as a new family of antileishmanial agents. In addition, benzoxadiazoles and their *N*-oxides have been previously studied as antibiotic and antiparasitic agents.[6] In this study, a facile synthesis route to carboxyimidamide-substituted benzoxadiazoles was developed, a set of 25 benzo[*c*][1,2,5]oxadiazole derivatives and other structurally related compounds was synthesized and evaluated as antileishmanial agents.

2. Results and discussion

2.1 Chemistry

Initially, benzo[*c*][1,2,5]oxadiazole-5-carbonitrile **4** was to be synthesized from 5-chloro- or 5-bromobenzo[*c*][1,2,5]oxadiazole in a microwave-assisted reaction using NaCN, CuCN, or $K_4[Fe(CN)_6]$,[7] but these

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Electronic Supplementary Information (ESI) available: Synthesis procedures for compounds **6-13**, **15-18**, **21-39**. See DOI: 10.1039/b000000x/

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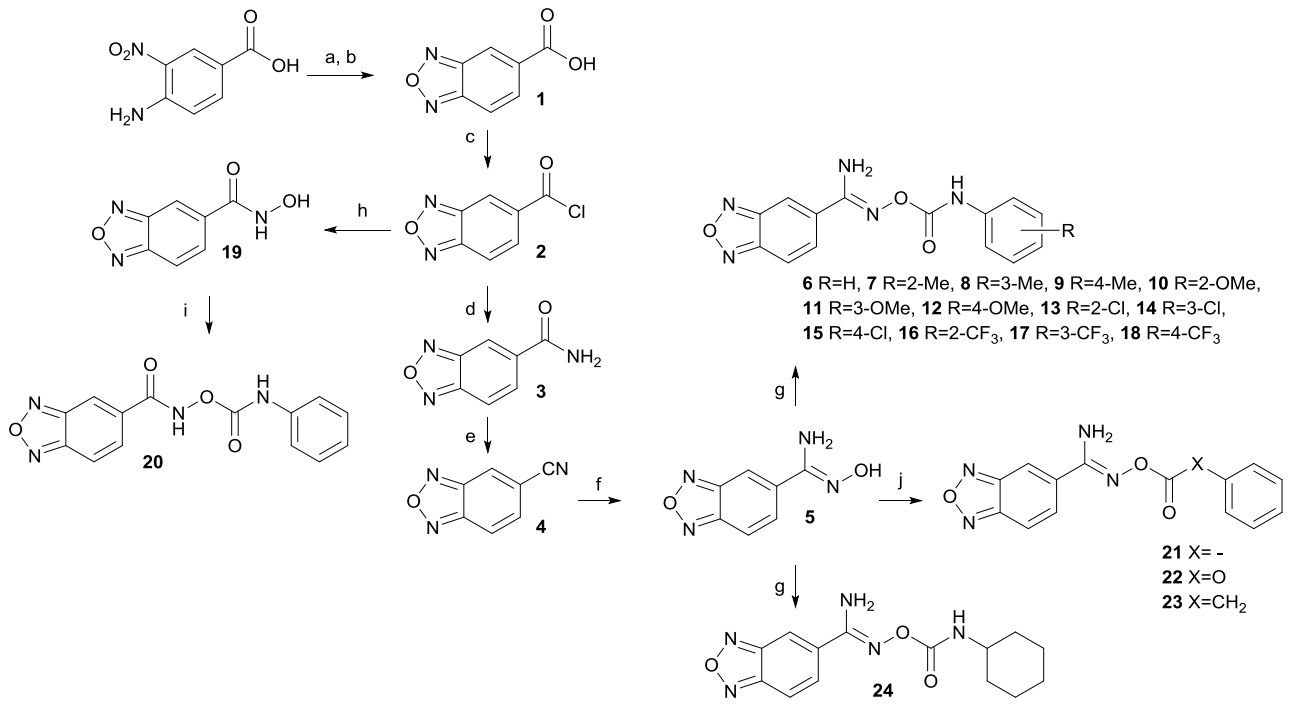
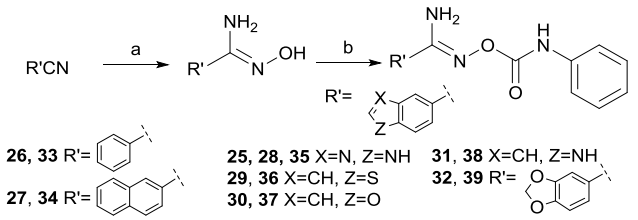


Scheme 1. Synthetic procedures and chemical structures of the products **2**-**24**. Reagents and conditions: a) KOH, EtOH, H_2O, 70 °C, 2 h, then NaOCl, 0 °C → rt, 21 h, 87%; b) $P(OEt)_3$, EtOH, 70 °C, 17 h, 67%; c) $SOCl_2$, reflux, 4 h, 97%; d) NH_3 aq., 1,4-dioxane, 0 °C, 1.5 h, 90%; e) TFAA, Et_3N, THF, rt, 3 h, 79%; f) $H_2NOH{\cdot}HCl$, Et_3N, EtOH, rt, 2 h, 94%; g) substituted phenyl isocyanate, THF or $CHCl_3$, rt, 3-21 or 96-120 h, 27-70%; h) MeOH, 0 °C, 0.5 h then $H_2NOH{\cdot}HCl$, MeOH, rt, 1.5 h, 57%; i) THF, phenyl isocyanate, 57%; j) PhCOCl/PhOCOCl/$PhCH_2COCl$, Et_3N, THF, 70 °C, 2-4 h, 14-63%; k) amine/benzylamine, Et_3N, THF, rt, 2 h, 29-53%.



Scheme 2. Synthetic procedures and chemical structures of the products **25**-**39**. Reagents and conditions: a) $H_2NOH{\cdot}HCl$, Et_3N or Na_2CO_3, EtOH or H_2O, rt/100 °C, 3.5-24 h, 38-98%; b), phenyl isocyanate, THF, $CHCl_3$ or 1,4-dioxane, rt, 2-5 h or 72 h, 6-85%.

syntheses failed to produce the target nitrile. Instead the synthesis of benzo[*c*][1,2,5]oxadiazole derivatives was started from the commercially available 4-amino-3-nitrobenzoic acid (Scheme 1). It was converted to benzo[*c*][1,2,5]oxadiazole-5-carboxylic acid (**1**) by using a two-step procedure via the *N*-oxide intermediate that was produced in the presence of sodium hypochlorite in an alkaline EtOH-H_2O solution and subsequently reduced to the carboxylic acid **1**.[8] Next, compound **1** was converted to the primary amide **3** via the acyl chloride **2** in the presence of aqueous ammonia in 1,4-dioxane. The reaction of the obtained primary amide **3** with trifluoroacetic anhydride and Et_3N in THF[9] gave the corresponding nitrile **4**, which was converted to the amidoxime **5** by using hydroxylamine hydrochloride[10] in the presence of Et_3N in EtOH. The final step to obtain the derivatives (**6**-**18, 24**) was carried out in the presence of the substituted phenyl isocyanates in THF or $CHCl_3$. The products **21**-**23** were synthesized in a similar manner by using a solution of Et_3N in THF. Compound **20** was synthesized from the acyl chloride **2** via the hydroxamic acid **19**. The final products **33**-**39** were synthesized from the commercially available nitriles by using the methods described above (Scheme 2). Finally, the related benzimidazole derivative **25** was formed from 3,4-diaminobenzonitrile in refluxing formic acid.[11]

2.2 Evaluation of antileishmanial activity

The activity of the new compounds was determined by using a fluorescent viability microplate assay with *L. donovani* axenic amastigotes and alamarBlue.[12] All compounds were initially screened at 50 μM, and the most active derivatives sequentially tested at lower concentrations (15 and 5 μM).

First, a set of benzo[*c*][1,2,5]oxadiazole derivatives **6**-**18**, with different substitution patterns in the "Eastern" phenyl ring of the structure, were synthesized and assayed. The derivative with no additional substituents (compound **6**) showed 70% and 24% inhibition of *L. donovani* proliferation at 50 μM and 15 μM, respectively, whereas *meta*-Cl substitution (compound **14**) increased growth inhibition to 95% and 87% at 50 μM and 15 μM, respectively (Table 1). Overall, *meta* substitution including Cl-, OMe-, Me-, and CF_3-substituted derivatives seems to be beneficial for antileishmanial activity.

Replacing HN of the heteroatom containing chain with oxygen or carbon atom (compounds **22** and **23**) significantly

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reduced antileishmanial activity to 26.5 and 22.4%, respectively, at 50 µM. Similarly, removal of the aniline NH (compound **21**) reduced activity, but this compound still showed more activity (51% growth inhibition) than carboxy **22** or benzylic **23** derivatives. Moreover, the cyclohexane derivative **24** displays leishmanial inhibition in the absence of an aniline moiety. Replacement of the amino group in the imidamide **20** with a carbonyl group increased inhibition to 96% and 68% at concentrations of 50 µM and 15 µM, respectively.

Table 1. Inhibition of *Leishmania donovani* axenic amastigote growth.

Compound	% Inhibition[a]		
	50 µM	15 µM	5 µM
6	70.1	24.2	—
7	42.0	—	—
8	67.2	44.6	—
9	64.1	—	—
10	38.0[b]	—	—
11	71.4	31.8	—
12	46.0	—	—
13	29.6[b]	—	—
14	94.8	87.2	43.4
15	44.0	—	—
16	42.1	—	—
17	51.4	—	—
18	51.7	—	—
20	96.2	68.2	54.6
21	51.1	—	—
22	26.5	—	—
23	22.4	—	—
24	43.9[b]	—	—
33	78.4	5.4	—
34	98.8	24.9	—
35	72.0	—	—
36	97.9	35.2	—
37	95.2	48.0	—
38	91.9	21.6	—
39	93.0	33.9	—
Amphotericin B	—	—	99.7[c]

[a] ± SEM: at 50 µM 0.1-2.3%, 15 µM 0.2-1.9%, and 5 µM 2.4-5.9%.
[b] precipitates at 50 µM.
[c] amphotericin B is positive control and was tested at 1 µM.

Finally, a second set of related heterocyclic compounds was synthesized with replacements of the benzo[*c*][1,2,5]oxadiazole moiety. These heterocycles (benzothiophene **36**, benzofuran **37**, indole **38**, and 1,3-benzodioxole **39**) showed an increase in antileishmanial activity at 50 µM compared to the benzoxazole derivative **6**. This suggests that the benzo[*c*][1,2,5]oxadiazole ring is not crucial to antileishmanial activity. In addition, the phenyl and naphthalene derivatives **33** and **34** significantly inhibited growth of *L. donovani* amastigotes (78% and 5% for **33**, and 99% and 25% for **34**, at concentrations of 50 µM and 15 µM, respectively). Good inhibition of compound **34** can be hypothesized to result from increased lipophilicity of the compound. Moreover, the phenyl derivative **33** is a very interesting compound, because the shorter synthetic route is likely to increase the prospects for further preparation of new antileishmanial derivatives of this chemotype.

Table 2. EC_{50} of the selected derivatives on *Leishmania donovani* axenic amastigotes and intracellular amastigotes in *L. donovani* infected macrophages; cytotoxicity on THP-1 macrophages and NIH/3T3 fibroblasts; and respective selectivity indices [SI].

Compound	Axenic amastigotes EC_{50} ± SEM (n=2) (µM)	Intracellular amastigotes EC_{50} ± SEM (n=3) (µM)	THP-1 EC_{50} (µM) (n=2)[SI]	Fibroblasts EC_{50} (µM) (n=2) [SI]
14	4.2±0.2	5.92±1.7	79.9±5.4 [19.0]	>300 [>71.4]
20	8.1±0.9	—	33.7±5.0 [4.2]	>300 [>37.0]
Ampho-tericin B	0.09-0.7 [12, 14-16]	0.11-0.26[17]	2.1-7.6 [15, 16]	2.2[18]

Further evaluation of the most promising derivatives **14** and **20** revealed low EC_{50} values on axenic amastigotes, 4.2 and 8.1 µM respectively; moderate cytotoxicity on the human THP-1 macrophage cell line, 79.9 µM and 33.7 respectively; and no cytotoxicity against murine fibroblasts at the highest concentration tested, 300 µM (Table 2). Values for amphotericin B, a reference compound used to treat leishmaniasis, are given for comparison. When tested at 5 µM on infected macrophages, a concentration non-toxic for the macrophage cell line, both compounds, **14** and **20**, were still active, 33.1 and 19.9% amastigote growth inhibition respectively, albeit lower than that observed with axenic amastigotes. The EC_{50} for **14**, the most active and least cytotoxic derivative, on intracellular amastigotes in infected macrophages was 5.92 µM similar to that observed using axenic amastigotes.

3. Conclusion

The developed synthesis route to carboxyimidamide-substituted benzo[*c*][1,2,5]oxadiazoles and related derivatives facilitates access to a compound library and determination of the structure-activity relationships for this antileishmanial heterocyclic chemotype. Carboxyimidamide-substituted benzoxadiazole derivative **14** is the most promising compound of this study, demonstrating good antileishmanial inhibition activity in infected macrophages and, remarkably, no signs of cytotoxicity. Although mechanism of action of the compounds is not known yet, it is worth continuing the development of these compounds to explore further prospects of these compounds.

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4. Experimental section

4.1 Chemistry

Unless otherwise stated, reactions were carried out in oven-dried glassware under an argon atmosphere. All reagents were commercially available and were acquired from Fluka (Buchs, Switzerland), Aldrich (Schnelldorf, Germany), Riedel-de Haën (Seelze, Germany), and Alfa Aesar (Karlsruhe, Germany). THF was distilled over sodium/benzophenone ketyl. The progress of chemical reactions was monitored by thin-layer chromatography on silica gel 60-F_{254} plates acquired from E. Merck (Darmstadt, Germany). The eluent consisted of EtOAc and *n*-hexane, or EtOAc and MeOH, and detection was conducted at 254 or 366 nm. The products were purified by flash chromatography on silica gel with a Biotage SP1 purification system (Uppsala, Sweden) using 25+M cartridges or SNAP 10 g, 25 g, or 50 g cartridges, detection at 254 nm. Melting points were measured using an IA9100 digital melting point apparatus (Electrothermal Engineering, Essex, UK) and are uncorrected. IR spectra were recorded on a Bruker Vertex 70 FT-IR spectrometer (Ettlingen, Germany) with ATR technique. The synthesized compounds were analyzed by NMR on a Varian Mercury 300 MHz spectrometer (Palo Alto, CA, USA). ^{1}H and ^{13}C NMR were recorded as solutions in DMSO-d_6 (Aldrich). Chemical shifts (δ) are given in parts per million (ppm) relative to the NMR solvent signals (DMSO-d_6 2.50 and 39.51 ppm for ^{1}H and ^{13}C NMR, respectively). LC-MS analyses were performed with a HP1100 instrument (Agilent, Palo Alto, USA) with UV detector (λ 210 nm) and an Esquire LC spectrometer (Bruker Daltonik, Bremen, Germany) with ESI ion source. Signal separation was carried out by the use of a Waters XBridge C18 column (2.1 mm × 50 mm, 2.1 µm) with a Waters XBridge C18 guard column (Milford, MA, USA) (2.1 mm × 10 mm, 2.5 µm). The eluent consisted of water (+ 0.1% HCO_2H) and acetonitrile (+ 0.1% HCO_2H) (gradient run 80:20 → 5:95). Purity of all tested compounds was >95%. High resolution mass spectra (HRMS) were measured on a Synapt G2 HDMS Q-TOF-instrument (Milford, MA, USA) with positive mode ESI.

Benzo[*c*][1,2,5]oxadiazole-5-carboxylic acid (1). Potassium hydroxide (2.02 g, 36.0 mmol, 2.4 equiv) was dissolved in equimixture of EtOH and H_2O (40 mL). 4-Amino-3-nitrobenzoic acid (2.73 g, 15.0 mmol) was added to this solution, and the resulting mixture was heated at 70 °C for 2 h and cooled to 0 °C. A 13% aqueous solution of sodium hypochlorite (80 mL) was added dropwise to this solution during 30 min. The resulting mixture was slowly allowed to warm to rt and stirred for 21 h. H_2O (35 mL) was added at 0 °C, and the mixture was acidified (pH <1) with a 6 M aqueous solution of HCl resulting in the formation of a yellow precipitate. Sodium chloride (1.9 g) was added, and the mixture was further stirred for 1.5 h, filtered, and dried *in vacuo* to yield benzo[*c*][1,2,5]oxadiazole-5-carboxylic acid 1-oxide (2.4 g, 87%) as a yellow solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.06 (br s, 1H), 7.84 (br s, 1H), 7.73 (br s, 1H). A mixture of benzo[*c*][1,2,5]oxadiazole-5-carboxylic acid 1-oxide (1.90 g, 10.5 mmol) in absolute EtOH (25 mL) was heated to 70 °C, and triethyl phosphite (2.80 mL, 16.0 mmol, 1.5 equiv) was added dropwise to the mixture. The resulting mixture was heated to reflux and the reflux was maintained for 17 h. The solvent was evaporated, and the residual black oil was treated with H_2O and extracted twice with EtOAc. The organic phase was extracted with a 2 M aqueous solution of NaOH, and the aqueous phase was washed three times with EtOAc. The aqueous phase was cooled to 0 °C and acidifed (pH <1) with a 6 M aqueous solution of HCl resulting in the formation of a light brown precipitate, which was filtered and dried *in vacuo* to yield the compound **1** (1.10 g, 67%) as a brown solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 13.7 (br s, 1H), 8.63 (t, J = 1.1 Hz, 1H), 8.13 (dd, J = 9.4, 1.1 Hz, 1H), 7.97 (dd, J = 9.4, 1.1 Hz, 1H). ^{13}C NMR (75 Hz, DMSO-d_6) δ 165.6, 149.3, 148.9, 134.7, 131.4, 119.4, 116.6. ^{1}H spectral data is consistent with those reported earlier.[14]

Benzo[*c*][1,2,5]oxadiazole-5-carbonyl chloride (2). A mixture of benzo[*c*][1,2,5]oxadiazole-5-carboxylic acid **1** (1.82 g, 11.1 mmol) and thionyl chloride (18 mL) was refluxed for 4 h. The resulting mixture was concentrated and dried *in vacuo* to yield **2** (2.0 g, 97%) as a brown solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.62 (t, J = 1.1 Hz, 1H), 8.12 (dd, J = 9.4, 1.1 Hz, 1H), 7.96 (dd, J = 9.4, 1.1 Hz, 1H). ^{13}C NMR (75 Hz, DMSO-d_6) δ 165.4, 149.3, 148.9, 134.6, 131.3, 119.4, 116.6.

Benzo[*c*][1,2,5]oxadiazole-5-carboxamide (3). A 25% aqueous solution of ammonia (4.8 mL) was cooled in an ice bath. To this solution, compound **2** (0.700 g, 3.80 mmol) in 1,4-dioxane (8 mL) was added dropwise. The resulting mixture was stirred at the same temperature for 1.5 h and then evaporated *in vacuo*. The residue was dissolved in EtOAc and washed with H_2O. The aqueous phase was extracted twice with EtOAc. The combined organic phases were dried over anhydrous Na_2SO_4, evaporated, and dried *in vacuo* to yield **3** (0.56 g, 90%) as a light orange solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.55 (t, J = 1.2 Hz, 1H), 8.32 (br s, 1H), 8.12 (dd, J = 9.4, 1.2 Hz, 1H), 7.97 (dd, J = 9.4, 1.2 Hz, 1H), 7.79 (br s, 1H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 166.0, 149.1, 148.9, 137.8, 131.4, 116.2, 115.8.

Benzo[*c*][1,2,5]oxadiazole-5-carbonitrile (4). Compound **3** (1.50 g, 9.20 mmol) was dissolved in anhydrous THF (30 mL), and distilled Et_3N (2.80 mL, 20.2 mmol, 2.2 equiv) was added to this solution. The solution was cooled in an ice bath, and trifluoroacetic anhydride (1.50 mL, 11.0 mmol, 1.2 equiv) in THF (8 mL) was added dropwise. The resulting mixture was stirred at rt for 3 h. H_2O was added and the volatiles were removed *in vacuo*. The residue was dissolved in CH_2Cl_2 and washed twice successively with a 0.1 M aqueous solution of HCl and a 0.1 M aqueous solution of NaOH. The organic phase was dried over anhydrous Na_2SO_4, evaporated, and dried *in vacuo*. The crude product was purified by flash column chromatography (EtOAc/*n*-hexane 1:2) to give **4** (1.10 g, 79%) as a pink solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.98 (t, J = 1.2 Hz, 1H), 8.28 (dd, J = 9.3, 1.2 Hz, 1H), 7.82 (dd, J = 9.3,

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1.2 Hz, 1H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 148.4, 147.9, 131.8, 125.7, 118.3, 117.2, 115.4.

***N*'-Hydroxybenzo[*c*][1,2,5]oxadiazole-5-carboximidamide (5).** A mixture of compound **4** (1.05 g, 7.20 mmol), $H_2NOHHCl$ (750 mg, 10.8 mmol, 1.5 equiv) and distilled Et_3N (1.50 mL, 10.8 mmol) in EtOH (45 mL) was stirred at rt for 2 h. The solvent was evaporated and the residue was dissolved in EtOAc and brine. The organic phase was washed with brine, dried over anhydrous Na_2SO_4, evaporated, and dried *in vacuo* to yield **5** (1.2 g, 94%) as a yellowish solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.38 (s, 1H), 8.29 (t, J = 1.1 Hz, 1H), 8.03 (dd, J = 9.6, 1.1 Hz, 1H), 7.96 (dd, J = 9.6, 1.1 Hz, 1H), 6.13 (s, 2H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 149.2, 149.0, 148.7, 136.4, 130.9, 115.2, 110.7.

***N*'-[[(3-Chlorophenyl)carbamoyl]oxy]benzo[*c*][1,2,5]oxadiazole-5-carboximidamide (14).** A mixture of **5** (89.0 mg, 0.500 mmol), and isocyanate (0.550 mmol, 1.1 equiv) in dry THF (5 mL) was stirred at rt for 4 h. The solvent was evaporated. The crude product was purified by flash SiO_2 column chromatography (EtOAc/*n*-hexane 1:2) to give **14** (0.10 g, 60%) as a white powder. ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.68 (s, 1H), 8.57 (s, 1H), 8.14-8.13 (m, 2H), 7.73-7.71 (m, 1H), 7.52 (dd, J = 8.4, 1.2 Hz), 7.36 (t, J = 8.4 Hz, 1H), 7.18 (s, 2H), 7,12 (dd, J = 8.4, 1.2 Hz, 1H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 153.6, 152.1, 148.9, 148.8, 140.0, 134.9, 133.1, 131.4, 130.4, 122.8, 118.8, 117.8, 116.0, 114.6. LC-MS: $[M+H]^+$ 332.0 *m/z* (t_r = 6.9 min). FT-IR (ATR, cm^{-1}): 3440, 3349, 1739, 1647, 1274. HRMS (ESI): *m/z* calcd for $C_{14}H_{10}ClN_5O_3$ $[M+H]^+$ 332.0550, found 332.0551.

***N*-Hydroxybenzo[*c*][1,2,5]oxadiazole-5-carboxamide (19).** Compound **2** (183 mg, 1.00 mmol) was stirred in MeOH (5 mL) in an ice bath for 30 min. The solvents were evaporated *in vacuo* to give methyl benzo[*c*][1,2,5]oxadiazole-5-carboxylate. A solution of hydroxylamine in MeOH was prepared by dissolving $H_2NOHHCl$ (542 mg, 7.80 mmol, 7.8 equiv) in MeOH (5 mL) in a water-ice bath. KOH (645 mg, 11.5 mmol, 11.5 equiv) in MeOH (10 mL) was added, resulting in formation of a precipitate. The resulting mixture was stirred in an ice-water bath for 30 min, and the precipitate was filtered off to yield a solution of *N*-hydroxycarboxamide in MeOH. The methyl ester (*vide supra*) was dissolved in this solution, stirred for 1.5 h, neutralized with acetic acid, and evaporated. The resulting residue was dissolved in EtOAc and washed with brine. The aqueous phase was further extracted with EtOAc. The combined organic phases were dried over with anhydrous Na_2SO_4, evaporated, and dried *in vacuo*. The crude product was purified by flash SiO_2 column chromatography (CH_2Cl_2/MeOH 9:1) to yield **19** (0.10 g, 57%) as a light solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 11.56 (br s, 1H), 9.40 (br s, 1H), 8.37 (s, 1H), 8.14 (dd, J = 9.4, 1.0 Hz, 1H), 7.88 (dd, J = 9.4, 1.0 Hz, 1H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 161.7, 149.0, 148.7, 139.6, 131.0, 116.5, 114.8.

***N*-[(Phenylcarbamoyl)oxy]benzo[*c*][1,2,5]oxadiazole-5-carboxamide (20).** A mixture of **19** (128 mg, 0.70 mmol) and phenyl isocyanate (87 µL, 0.77 mmol, 1.1 equiv) in dry THF (5 mL) was stirred at ice-water bath temperature for 30 min. The resulting mixture was let warm to rt, stirred for 3 h, evaporated, and dried *in vacuo*. The crude product was purified by flash SiO_2 column chromatography (CH_2Cl_2/MeOH 19:1) to yield **20** (0.12 g, 57%) as a light brownish solid. ^{1}H NMR (300 MHz, DMSO-d_6) δ 12.79 (br s, 1H), 10.38 (br s, 1H), 8.55 (t, J = 1.2 Hz, 1H), 8.22 (dd, J = 9.5, 1.2 Hz, 1H), 7.92 (dd, J = 9.5, 1.2 Hz, 1H), 7.52-7.49 (m, 2H), 7.37-7.32 (m, 2H), 7.11-7.05 (m, 1H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 163.0, 152.0, 149.1, 148.6, 138.0, 134.9, 130.6, 129.0, 123.3, 118.5, 117.1, 116.7. LC-MS: $[M+H]^+$ 299.2 *m/z* (t_r = 5.2 min). FT-IR (ATR, cm^{-1}): 3321, 3224, 1759, 1663, 1535, 1203, 888. HRMS (ESI): *m/z* calcd for $C_{14}H_{11}N_4O_4$ $[M+H]^+$ 299.0780, found 299.0779.

4.2 Biology

Antileishmanial evaluation using axenic amastigotes *L. donovani* (MHOM/SD/1962/1S-Cl2d) was used in all bioassays. Screening of the compounds for antileishmanial activity using axenic amastigotes was carried out using the alamarBlue (AbD Serotec, Oxford, UK) viability assay, as previously described, similar to that reported for leishmanial promastigotes.[12] Axenic amastigotes were grown at 37 °C in a 5% CO_2 incubator in complete RPMI 1640 containing 20% fetal calf serum, pH 5.5.[13] Compounds to be assayed were diluted in the complete amastigote medium containing 1% DMSO to twice the final concentration used in the assays, and were aliquoted in triplicate (125 µL/well) into 96-well flat-bottom plates (Nunc, Roskilde, Denmark). For determination of the EC_{50}, final concentrations in triplicate spanning 300 to 0.14 µM were used. Amastigotes (5.0×10^5 cells/mL; 125 µL/well) were added to each well and incubated for 24 h at 37 °C in a 5% CO_2 incubator. The alamarBlue viability indicator was added (25 µL/well) and the plates incubated for an additional 24 h at which time the fluorescence (λ_{ex} = 544 nm; λ_{em} = 590 nm) was measured in a microplate reader (Fluoroskan Ascent FL, Finland). Complete medium, both with and without DMSO, was used as negative controls (0% inhibition of amastigote growth). Amphotericin B (Sigma-Aldrich, St. Louis MO), a drug used to treat VL, was included as a positive control on each plate and gave >90% inhibition of parasite growth at 1.5 µM.

Cytotoxicity EC_{50} was determined on NIH/3T3 fibroblasts or THP-1 monocyte cells using the alamarBlue viability indicator as follows. Cells in DMEM medium plus 10% fetal calf serum and antibiotics were aliquoted in triplicate (fibroblast or THP-1 at 8×10^5 or 6.4×10^4 cells/well respectively; 125 µL/well) into 96-well plates (Nunc, Roskilde, Denmark). Compounds to be assayed were diluted in the same medium containing 1% DMSO at twice the final concentration used in the assays (300 to 0.14 µM for fibroblasts or 150 to 0.10 µM for THP-1), added to the cells (125 µL/well), and the plates incubated for 48 h at 37 °C in a 5% CO_2 incubator. The alamarBlue viability indicator was added (25 µL/well) and the plates incubated for an additional 3-5 h at which time the fluorescence (λ_{ex} = 544 nm; λ_{em} = 590 nm) was measured. Complete medium both with

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and without DMSO was used as negative controls (0% inhibition).

Activity on intracellular amastigotes Inhibition of amastigote growth in infected THP-1 cells was measured as previously described.[5] In brief, THP-1 cells in complete RPMI-1640 containing 10% fetal calf serum and antibiotics were treated for 24 h with 25 ng/mL phorbol 12-myristate-13-acetate (PMA, Sigma-Aldrich, St. Louis, MO). PMA and non-adherent cells were removed with excess warm medium, and stationary-phase *Ld*:pSSU-int/LUC promastigotes expressing luciferase (5:1 parasite/macrophage ratio) used to infect the macrophages. Twenty-four hours later the adherent cells were washed 4-5 times with warm RPMI-1640 alone, and infected macrophages detached by treating with Trypsin EDTA. Infected macrophages (5×10^4 cells/100 µL/well) were dispensed in triplicate into white 96-well flat bottom plates (NUNC, Denmark) and the compounds diluted in complete RPMI-1640 containing 1% DMSO final concentration and added in triplicate (50 µL/well). Cultures were incubated for a further 48 h (37 °C, 5% CO_2), lysed by the addition of Bright-Glo Luciferase Assay substrate (100 µL/well, Promega, MT, U.S.A.), and chemiluminescence measured using a microplate reader (Fluoroskan Ascent, Thermo Scientific). Amphotericin B (1 µM, >90% inhibition of parasite growth) was included as a positive control. Complete medium both with and without DMSO were used as negative controls. Experiments were repeated three times. Calculation of the EC_{50}'s and statistical analysis were carried out using GraphPad Prism Version 6.0b (GraphPad Software, Inc. San Diego, CA).

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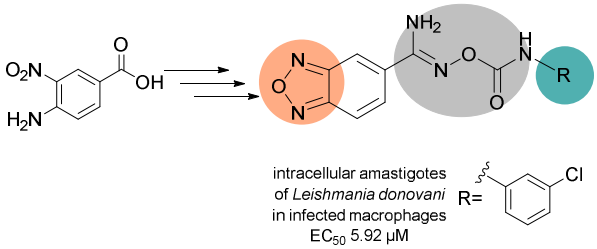
Table of contents entry (graphical abstract and 20-30 words of text)

Facile synthesis route to carboxyimidamide-substituted benzo[*c*][1,2,5]oxadiazole and related derivatives was developed, and 25 derivatives were synthesized. Antileishmanial evaluation revealed promising inhibition activity.



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