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Antikinetoplastid activity of 3-aryl-5-thiocyanatomethyl-1,2,4-oxadiazoles

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Abstract—A series of 5-thiocyanatomethyl- and 5-alkyl-3-aryl-1,2,4-oxadiazoles were synthesized and evaluated for their activity against kinetoplastid parasites. Formation of the oxadiazole ring was accomplished through the reaction of benzamidoximes with acyl chlorides, while the thiocyanate group was inserted by reacting the appropriate 5-halomethyl oxadiazole with ammonium thiocyanate. The thiocyanate-containing compounds possessed low micromolar activity against *Leishmania donovani* and *Trypanosoma brucei*, while the 5-alkyl oxadiazoles were less active against these parasites. 3-(4-Chlorophenyl)-5-(thiocyanatomethyl)-1,2,4-oxadiazole (compound **4b**) displayed modest selectivity for *L. donovani* axenic amastigote-like parasites over J774 macrophages, PC3 prostate cancer cells, and Vero cells (6.4-fold, 3.8-fold, and 9.1-fold, respectively), while 3-(3,4-dichlorophenyl)-5-(thiocyanatomethyl)-1,2,4-oxadiazole (compound **4h**) showed 30-fold selectivity against Vero cells but was not selective against PC3 cells. In a murine model of visceral leishmaniasis, compound **4b** decreased liver parasitemia caused by *L. donovani* by 48% when given in five daily i.v. doses at 5 mg/kg and by 61% when administered orally for 5 days at 50 mg/kg. These results indicate that aromatic thiocyanates hold promise for the treatment of leishmanial infections if the selectivity of these compounds can be improved.

1. Introduction

Leishmaniasis and African trypanosomiasis continue to place a heavy burden on millions of people in developing nations. Both are vector-borne diseases caused by protozoan parasites of the phylogenetic order Kinetoplastida. Depending on the causative *Leishmania* species, leishmaniasis presents as a spectrum of disease ranging from self-resolving cutaneous infections to life-threatening visceral manifestations. *Trypanosoma brucei rhodesiense* and *Trypanosoma brucei gambiense* parasites give rise to acute and chronic central nervous system infections, respectively, that are ultimately fatal in the absence of treatment. Further information regarding the transmission, symptoms, geographical distribution, and prevalence of these diseases can be found on websites maintained by the World Health Organization (see http://www.who.int/inf-fs/en/fact116.html and http://www.who.int/inf-fs/en/fact259.html). Unfortunately, the drugs that are commonly used against leishmaniasis and African trypanosomiasis are administered by injection, are moderately to extremely toxic, and have been compromised by the development of resistance in some areas (see Refs. 1 and 2 for recent reviews). The registration of miltefosine in India as the first oral treatment for visceral leishmaniasis[3] and ongoing clinical trials for the orally available pentamidine analog DB289 as a candidate for the treatment of early stage African trypanosomiasis[4] provide hope that improved drugs for these diseases may soon be within reach. The widespread utility of these agents is not yet established, however, and the chemotherapeutic armamentarium against kinetoplastid parasites remains limited. New drug candidates are clearly needed in the fight against these pathogens.

Recent work in our laboratory has focused on the investigation of tubulin as an antikinetoplastid drug

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target.[5,6] In the context of these studies, we found that an oxadiazole-containing aromatic thiocyanate from the Walter Reed Chemical Inventory, 3-(4-chlorophenyl)-5-(thiocyanatomethyl)-1,2,4-oxadiazole (WR85915), possessed intriguing antileishmanial activity.[7] This compound blocked the assembly of leishmanial tubulin in vitro, presumably by binding covalently to one or more sulfhydryl residues of the protein,[8] and inhibited the growth of *L. donovani* axenic amastigote-like parasites by 50% at a concentration of 4.3 μM.[7] Cellular studies with WR85915 suggested that microtubule disruption was not the primary mechanism of action of this compound in *Leishmania*, however, indicating that one or more other targets in the parasite are affected.[7] An independent report appearing at about the same time as our work also demonstrated that three simple aromatic thiocyanates possess micromolar activity against the related kinetoplastid parasite *Trypanosoma cruzi*, the causative agent of Chagas' disease.[9] Antimonial and arsenical drugs, which have long been the mainstays of antikinetoplastid chemotherapy, react readily with sulfhydryl residues in their +3 oxidation states. Wild-type *L. donovani* amastigotes reduce Sb(V) to the thiol-reactive Sb(III) species, but organisms resistant to the Sb(V)-containing antileishmanial drug Pentostam do not perform this reduction.[10] Given the known activity of sulfhydryl-reactive compounds against kinetoplastid parasites and the promising in vitro activity of aromatic thiocyanates against these organisms, we chose to prepare analogs of the oxadiazole-containing thiocyanate WR85915 and examine their activity against *Leishmania* and African trypanosomes.

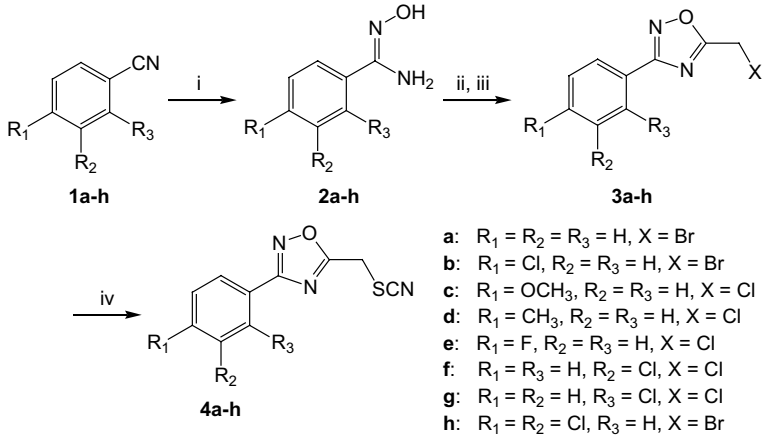
2. Results and discussion

2.1. Chemistry

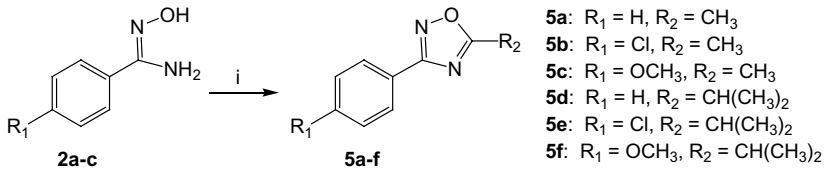
The desired thiocyanates were prepared as outlined in Scheme 1. Conversion of **1a**–**h** into benzamidoximes **2a**–**h** was accomplished by treatment of the benzonitriles with hydroxylamine hydrochloride in ethanol in the presence of the chelating agent 8-hydroxyquinoline.[11] Inclusion of 8-hydroxyquinoline in these reactions decreased the fraction of the unwanted benzamide side product, presumably by chelating trace metal ions as suggested by Yamanaka et al.[11] Benzamidoximes **2a**–**h** were converted to 3-aryl-5-halomethyl oxadiazoles **3a**–**h** in a two-step reaction sequence. Treatment of **2a**–**h** with either chloroacetyl chloride, bromoacetyl bromide, or bromoacetyl chloride in the presence of equimolar potassium carbonate in acetone solvent afforded O-acyl adducts that were subsequently converted to the halomethyl oxadiazoles in good to excellent overall yields by heating at reflux in toluene.[12] Procedures employing chloroacetyl chloride or bromoacetyl bromide were preferable to those using bromoacetyl chloride because exchange of chlorine for bromine led to the formation of a mixture of bromomethyl and chloromethyl oxadiazoles. Halomethyl oxadazoles **3a**–**h** were transformed into the corresponding methyl thiocyanates **4a**–**h** in fair to good yield by treatment of **3a**–**h** with ammonium thiocyanate in DMF. This protocol was based on previous procedures where thiocyanatomethyl oxadiazoles were prepared from halomethyl oxadiazoles using potassium thiocyanate or ammonium thiocyanate in DMSO.[13,14] 5-Alkyl oxadiazoles were synthesized as shown in Scheme 2. Transformation of benzamidoximes **2a**–**c** into the 3-aryl-5-alkyl-1,2,4-oxadiazoles **5a**–**f** was readily accomplished by treatment of **2a**–**c** with acetyl or isobutyryl chloride in pyridine.[15]

2.2. Antiparasitic activity

We first investigated the antiparasitic activities of the 5-thiocyanotomethyl- and 5-alkyl-3-aryl-1,2,4-oxadiazoles against *Leishmania donovani* axenic amastigote-like parasites in vitro. In the series of 5-thiocyanotomethyl-3-aryl-1,2,4-oxadiazoles, different substituents were introduced on the 3-aryl ring in order to assess the effects of lipophilicity, steric bulk, and electronic factors on antiparasitic activity at that site. The thiocyanate-containing compounds **4a**–**h** displayed antileishmanial IC_{50} values at concentrations ranging from 2.3 to 12 μM. Although this range of IC_{50} values is rather narrow, the



Scheme 1. Reagents and conditions: (i) hydroxylamine hydrochloride, 8-hydroxyquinoline, EtOH; (ii) haloacetyl halide, K_2CO_3, acetone; (iii) toluene, reflux; (iv) ammonium thiocyanate, DMF.



Scheme 2. Reagents and conditions: (i) acetyl or isobutyryl chloride, pyridine.

order of antileishmanial activity in the series **4h** (3,4 di-Cl) > **4b** (4-Cl) > **4d** (4-CH_3) > **4c** (4-OCH_3) > **4a** (4-H) suggests that greater lipophilicity in the 3-aryl ring augments antileishmanial potency in this group of thiocyanate-containing oxadiazoles.[16] The in vitro antileishmanial activities of **4b**, **4e**, and **4h** approach that of the clinically useful antileishmanial drug pentamidine, indicating that these aromatic thiocyanates were worthy of further investigation. Oxadiazoles bearing 5-alkyl substitutions (**5a**–**f**) were ineffective against *Leishmania*, strongly suggesting that the 5-thiocyanatomethyl group is essential for antikinetoplastid activity in this group of molecules. Aromatic thiocyanates **4a**–**h** were also tested against bloodstream-form *Trypanosoma brucei*. Little difference in antitrypanosomal potency was observed among the eight compounds, with IC_{50} values ranging from 5.2 to 7.4 μM. As with *L. donovani*, compounds **5a**–**f** exhibited weaker activity against *T. brucei*. Although **4a**–**h** affect the growth of both African trypanosomes and *Leishmania* at comparable concentrations in vitro, clinically effective antitrypanosomal drugs such as pentamidine and suramin display in vitro activity at nanomolar concentrations (see Table 1). Subsequent studies thus focused on the antileishmanial potential of the 5-thiocyanatomethyl-3-aryl-1,2,4-oxadiazoles.

The antileishmanial selectivity of **4a**–**h** was examined by evaluating the effect of these compounds on the growth of mammalian cell lines. Compounds **4b**, **4d**, **4f**, and **4h** showed modest in vitro selectivity against amastigote-like *L. donovani* compared to J774 murine macrophages, with these agents being 6.4-fold, 3.4-fold, and 4.3-fold, and 7.4-fold more active against *Leishmania* than the macrophage cell line, respectively. While compounds **4b** and **4d** displayed modest selectivity against PC3 prostate cancer cells, being 3.8-fold and 2.9-fold more potent against *L. donovani*, compounds **4f** and **4h** displayed less than 2-fold selectivity compared to this cancer cell line. When the two most active agents against *Leishmania* were examined against African Green Monkey kidney (Vero) cells, greater selectivity was observed (9.1-fold for **4b** and 30-fold for **4h**). Compound **4b**, which displayed modest but consistent selectivity across the three cell lines in vitro, was chosen for further antileishmanial evaluation in an intracellular model employing *L. mexicana*-infected J774 macrophages (Table 2). Although the IC_{50} value of **4b** against J774 macrophages is reported as 29 μM in Table 1, 50 μM concentrations of **4b** could be employed in this intracellular antileishmanial assay without obvious toxicity to the mammalian cells because a 50-fold higher starting concentration of macrophages is used in the intracellular assay. Compared to controls, parasite burdens were decreased by about half at 50 μM **4b** and by approximately one-third at 25 μM **4b**, while 12.5 μM concentrations of this compound had essentially no effect on intracellular *L. mexicana*. Our measured activity of amphotericin B, the most consistent and effective standard compound in this assay, was comparable to the 40 nM ED_{50} for the reduction of amastigotes in peritoneal macrophages reported by Neal and Croft.[17]

While amphotericin B is clearly superior to **4b** in the *Leishmania*-infected macrophage assay, other clinically useful antileishmanial compounds such as pentamidine and Pentostam are much less active than amphotericin B

Table 1. IC_{50} values (μM) of 3-aryl-5-substituted-1,2,4-oxadiazoles against axenic *L. donovani*, *T. brucei*, and mammalian cell lines

Compound	*L. donovani* axenic amastigotes	*T. b. brucei* variant 221	J774 macrophages	PC3 prostate	Vero cells
4a	9.5 ± 2.5	7.4 ± 1.4	7.2 ± 0.4	6.2 ± 0.7	ND[a]
4b	4.5 ± 1.8	5.2 ± 1.7	29 ± 7	17 ± 6	41 ± 14
4c	7.3 ± 3.3	6.9 ± 0.3	8.7 ± 1.1	8.4 ± 4.2	ND
4d	6.2 ± 0.6	6.7 ± 0.3	21 ± 6	18 ± 8	ND
4e	4.9 ± 1.8	6.4 ± 0.5	9.5 ± 0.6	7.5 ± 3.2	ND
4f	6.5 ± 0.6	5.2 ± 1.7	28 ± 8	8.1 ± 1.1	ND
4g	12 ± 0	6.7 ± 0.2	11 ± 1	8.4 ± 3.9	ND
4h	2.3 ± 1.0	5.2 ± 2.0	17 ± 2	3.9 ± 2.2	70 ± 22
5a	>200	>25	ND	ND	ND
5b	>200	ND	ND	ND	ND
5c	>200	>25	ND	ND	ND
5d	>200	>25	ND	ND	ND
5e	>200	>25	ND	ND	ND
5f	>100	>25	ND	ND	ND
Pentamidine	1.1 ± 0.1	0.008 ± 0.006	23 ± 6	32 ± 14	>100
Suramin	ND	0.20 ± 0.05	ND	ND	ND

[a] ND—not determined.

Table 2. Activity of **4b** and amphotericin B against intracellular *L. mexicana*

Compound	Concentration (μM)	% Reduction in amastigotes/ macrophage
4b	50	48 ± 17
	25	36 ± 14
	13	2 ± 14
Amphotericin B	0.50	99 ± 0
	0.13	77 ± 25
	0.031	29 ± 16

in this intracellular model.[17] Considering the in vitro potency and selectivity of **4b**, we examined the activity of this compound in a rodent model of visceral leishmaniasis (Table 3). The activity of Pentostam observed here was comparable to the roughly 50% inhibition of liver parasitemia reported previously for an identical dose of this antimonial drug given by the same route.[18] When administered i.v. at a dose of 5 mg/kg, compound **4b** was slightly less active than a 15 mg/kg s.c. dose of Pentostam but more active than the highest tolerable dose of i.v.-administered pentamidine. In a separate experiment, **4b** suppressed liver parasitemia by 61% when given at 50 mg/kg in five daily oral doses. No toxicity was observed when **4b** was given at the 5 mg/kg i.v. dose, while a slight weight loss was observed in the group treated with the 50 mg/kg oral dose of this compound.

2.3. Conclusions

Compound **4b** (WR85915) displays good in vitro and in vivo activity against *Leishmania*. While the antiparasitic selectivity of such aromatic thiocyanates is clearly a concern, electrophilic molecules have a long history of clinical success in treating diseases caused by kinetoplastid parasites. The synthesis of aromatic thiocyanates such as **4b** is also relatively straightforward, suggesting that these types of molecules could be produced inexpensively for treating diseases that are prevalent in the developing world. Future studies seeking to identify the major antiparasitic target(s) of **4b** and aiming to increase the potency and specificity of analogs of this compound against these target(s) could prove to be a fruitful approach in the search for critically-needed antileishmanial agents.

3. Experimental

3.1. General methods

All reagents were obtained from commercial vendors and were used without further purification unless otherwise indicated. Nuclear magnetic resonance spectra were obtained at 250, 400, or 600 MHz for ^{1}H and 62.5, 100, or 150 MHz for ^{13}C using instruments from Bruker. Elemental analyses were performed by Atlantic Microlabs (Norcross, GA) and were within ±0.4% of the calculated values.

3.2. Benzonitriles 1a–h

With the exception of the costly 3,4-dichlorobenzonitrile (**1h**), which was prepared by the reaction of 3,4-dichlorobenzaldehyde with hydroxylamine and phthalic anhydride,[19] benzonitrile precursors were obtained from commercial sources.

3.3. Preparation of benzamidoximes 2a–h

Benzamidoximes were synthesized by the reaction of **1a**–**h** with hydroxylamine hydrochloride in the presence of sodium carbonate and 8-hydroxyquinoline. *Representative procedure for the preparation of 4-methylbenzamidoxime* **2d**. To a solution of **1d** (25.0 g, 0.21 mol) in ethanol (200 mL) was added 8-hydroxyquinoline (0.077 g, 0.53 mmol). Hydroxylamine hydrochloride (31.3 g, 0.45 mol) and sodium carbonate (35.8 g, 0.34 mol), each dissolved in water (100 mL), were sequentially added over a period of 20 min, then the reaction mixture was heated to reflux for 4.5 h. After cooling the mixture, solvent was removed in vacuo to give a light olive green solid. Chromatography on silica gel using ethyl acetate as eluent gave 31.6 g of a slightly yellow crystalline solid. This crude material was further purified by recrystallization from ethanol to give 17.8 g (56%) of the title compound as a white crystalline solid, mp 145–147 °C, lit. mp 146 °C.[20] Concentration of the mother liquors via aspirator afforded an additional 5.3 g (16%) of **2d**, mp 140–146 °C. Melting points for compounds **2a**–**c**,[20] **2e**,[21] and **2f**[21] were also consistent with those reported in the literature.

Table 3. Activity of **4b**, Pentostam, and pentamidine against *L. donovani* in BALB/c mice

Compound	Dosing regimen	Actual total dose received	% Inhibition ± S.E.M.
Experiment 1			
4b	5 mg/kg i.v. × 5	2.5 mg	48 ± 8
Pentostam	15 mg Sb(V)/kg s.c. × 5	7.5 mg Sb(V)	60 ± 5
Pentamidine	5 mg/kg i.v. × 5[a]	1.5 mg[a]	31 ± 5
Experiment 2			
4b	50 mg/kg p.o. × 5[b]	25 mg[b]	61 ± 8
Pentostam	15 mg Sb(V)/kg s.c. × 5	7.5 mg Sb(V)	47 ± 5

[a] Pentamidine produced irritation on administration of full dose on day 1. The dose was reduced by 50% for all mice for the following 4 days.
[b] A slight loss of weight in this group of mice was observed.

3.3.1. 2-Chlorobenzamidoxime 2g. Melting point 119–121 °C, lit. 116–117 °C.[21] ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.76 (br s, 1H), 7.52 (dd, $J_1 = 7.5$ Hz, $J_2 = 1.4$ Hz, 1H), 7.42 (m, 1H), 7.37–7.26 (m, 2H), 4.95 (s, 2H).

3.3.2. 3,4-Dichlorobenzamidoxime 2h. Melting point 129–135 °C, lit. 138.5–142 °C.[21] ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.85 (br s, 1H), 7.74 (m, 1H), 7.48 (m, 2H), 4.85 (s, 2H).

3.4. Preparation of halomethyl oxadiazoles

3.4.1. 3-Phenyl-5-(bromomethyl)-1,2,4-oxadiazole 3a. To a solution of **2a** (13.6 g, 0.10 mol) in chloroform (300 mL) was added sodium carbonate (10.6 g, 0.10 mol). Bromoacetyl chloride (21.3 g, 0.135 mol) in chloroform (9 mL) was added to the reaction mixture over a period of 75 min. A white solid suspended in the solution formed upon addition of the bromoacetyl chloride. The reaction mixture was stirred an additional 60 min, then solvent was removed in vacuo to give the crude product. The solid was dissolved in ethyl acetate (400 mL), then the organic layer was washed with water (3 × 100 mL), brine (3 × 100 mL), dried (Na_2SO_4), and filtered. Removal of the solvent by evaporation under vacuum gave 23.9 g (93%) of the O-acyl adduct as a white powder. This crude adduct (22.9 g, 0.089 mol) was suspended in 500 mL of *o*-xylene and refluxed for 3 h. Solvent was removed under vacuum from the hot reaction mixture to give the product as a yellow oil, which crystallized on standing. The crude product was dissolved in ethyl acetate (300 mL), then the organic layer was washed with water (3 × 100 mL), brine (3 × 100 mL), and dried (Na_2SO_4). The solution was filtered and solvent was evaporated to give 21.1 g of a slightly yellow solid. Chromatography on silica gel using ethyl acetate/hexane (1:4) gave 11.4 g (54%) of the title compound as a white crystalline solid, mp 47–52 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.11–8.08 (m, 2H), 7.56–7.48 (m, 3H), 4.57 (s, 2H). ^{13}C NMR (150 MHz, $CDCl_3$): δ 174.5, 168.9, 131.5, 128.9, 127.5, 126.2, 16.4. Note: Integration of the ^{1}H NMR spectrum indicated that 6% of the chloromethyl compound (singlet at 4.77 δ) had been formed by exchange of chlorine for bromine, which apparently took place under the reaction conditions used in the preparation of the precursor O-acyl adduct.

3.4.2. 3-(4-Chlorophenyl)-5-(bromomethyl)-1,2,4,-oxadiazole 3b. To a solution of **2b** (17.6 g, 0.10 mol) in methylene chloride (300 mL) was added potassium carbonate (13.8 g, 0.10 mol). Bromoacetyl chloride (21.1 g, 0.13 mol) was added dropwise over a period of 3 h. Solvent was evaporated to give the crude product as a white powder. The adduct was dissolved in ethyl acetate (500 mL), the organic layer was washed with water (3 × 300 mL) and brine (3 × 300 mL), then the ethyl acetate solution was dried over sodium sulfate and filtered. Evaporation of the solvent gave the crude product as a yellow solid. The material was chromatographed on silica gel with ethyl acetate/hexane (3:7) to give 24.0 g (83%) of the O-acyl adduct as a slightly yellow solid, mp 126–130 °C. A solution of this O-acyl adduct (24.0 g, 0.082 mol) in 500 mL of toluene was refluxed and stirred for 5 h. Solvent was removed under vacuum from the hot solution to give the crude product as a yellow solid. The product was dissolved in a minimum volume of ethyl acetate and chromatographed on silica gel with ethyl acetate/hexane (1:9). Elution of the column afforded 20.5 g (92%) of the title compound as a slightly yellow solid, mp 67–70 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.01 (d, $J = 8.7$ Hz, 2H), 7.46 (d, $J = 8.7$ Hz, 2H), 4.55 (s, 2H). ^{13}C NMR (150 MHz, $CDCl_3$): δ 174.8, 168.2, 137.7, 129.3, 128.8, 124.7, 16.3. Note: Integration of the ^{1}H NMR spectrum indicated that 4% of the chloromethyl compound (singlet at 4.74 δ) had been formed by exchange of chlorine for bromine, which apparently took place under the reaction conditions used in the preparation of the precursor O-acyl adduct.

3.4.3. 3-(4-Methoxyphenyl)-5-(chloromethyl)-1,2,4-oxadiazole 3c. To a solution of **2c** (14.0 g, 0.084 mol) in dry acetone (225 mL) was added potassium carbonate (11.6 g, 0.084 mol). Chloroacetyl chloride (8.07 mL, 11.3 g, 0.10 mol) was added dropwise over a period of 1.5 h to the stirred reaction mixture, and the reaction was allowed to stir overnight at room temperature. Solvent was then removed under reduced pressure to give the product as a white powder. This solid was dissolved in ethyl acetate (400 mL) and was partitioned once with water (100 mL). The organic layer was then washed with water (3 × 50 mL), brine (3 × 50 mL), dried over Na_2SO_4, and concentrated in vacuo to give 8.71 g (43%) of the product as a light yellow gummy solid. The O-acyl adduct (8.4 g, 0.035 mol) was added to 150 mL of toluene and was heated to reflux for 5 h. Removal of the solvent in vacuo gave 7.4 g of the product as a brown oil. The oil was chromatographed on silica gel using ethyl acetate/hexane (1: 9) as an eluent to afford 5.75 g of the product as an off-white solid. Recrystallization of the product from hexane gave 4.71 g (60%) of the title compound as a white crystalline solid, mp 53–56 °C (Note: this compound was previously listed as a liquid[12]). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.02 (d, $J = 8.9$ Hz, 2H), 6.99 (d, $J = 8.9$ Hz, 2H), 4.73 (s, 2H), 3.87 (s, 3H). ^{13}C NMR (150 MHz, $CDCl_3$): δ 174.0, 168.6, 162.2, 129.2, 118.6, 114.4, 55.4, 33.4.

3.4.4. 3-(4-Methylphenyl)-5-(chloromethyl)-1,2,4-oxadiazole 3d. To a solution of **2d** (17.10 g, 0.110 mol) in 250 mL of dry acetone was added potassium carbonate (15.2 g, 0.11 mol). Chloroacetyl chloride (9.61 mL, 13.6 g, 0.12 mol) was added dropwise over a period of 1 h to the stirred reaction mixture, and the reaction was allowed to stir overnight at room temperature. Solvent was then removed under reduced pressure to give the product as a white solid. This solid was dissolved in ethyl acetate (500 mL), the organic layer was washed with water (4 × 200 mL), brine (1 × 200 mL), dried over Na_2SO_4, and filtered. Solvent was removed in vacuo to give a white crystalline solid, mp 116–119 °C. The product was recrystallized from ethanol/chloroform/hexane (1:1:1) to

give 9.85 g (40%) of the O-acyl adduct as a white crystalline solid. The O-acyl adduct (8.50 g, 0.038 mol) was suspended in 200 mL of toluene, then the reaction mixture was heated to reflux and stirred for 5 h. Removal of the solvent in vacuo gave 7.81 g of the crude product as a light yellow oil, which crystallized on standing. This solid was chromatographed on silica gel with ethyl acetate/hexane (1:9) to afford 7.45 g of a white crystalline solid. The product was recrystallized from hexane to give 5.40 g (68%) of the title compound as a white crystalline solid, mp 46–48 °C, lit. 40–41 °C.[22] ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.96 (d, $J = 8.2$ Hz, 2H), 7.29 (d, $J = 7.9$ Hz, 2H), 4.73 (s, 2H), 2.41 (s, 3H). ^{13}C NMR (150 MHz, $CDCl_3$): δ 174.1, 168.9, 142.0, 129.7, 127.4, 123.4, 33.4, 21.6.

3.4.5. 3-(4-Fluorophenyl)-5-(chloromethyl)-1,2,4-oxadiazole 3e. To a stirred solution of **2e** (16.2 g, 0.105 mol) in 500 mL of dry acetone was added potassium carbonate (14.5 g, 0.105 mol). Over a period of 3 h, a solution of chloroacetyl chloride (8.3 mL, 11.8 g, 0.10 mol) in 10 mL of dry acetone was added dropwise to the reaction mixture. Stirring was continued for an additional 3 h at room temperature, then solvent was removed in vacuo from the reaction mixture to give an off-white solid. The solid was washed with water (4×100 mL), then was dissolved in ethyl acetate (400 mL). This organic layer was washed with water (4×100 mL), brine (4×100 mL), then dried over Na_2SO_4. Evaporation of the solvent gave 19.6 g (81%) of the O-acyl adduct. This O-acyl-adduct (16.3 g, 0.07 mol) was added to 250 mL of toluene, then the solution was heated to reflux with stirring for 6 h. Solvent was evaporated from the reaction mixture to give 15.8 g of the crude product as an oil. This oil was chromatographed on silica gel with ether/hexane (1:4) to afford the oxadiazole as a colorless oil, which solidified upon refrigeration. Recrystallization of the product from hexane afforded 13.1 g (88%) of the title compound as a white crystalline solid, mp 31–32 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.09 (m, 2H), 7.18 (m, 2H), 4.75 (s, 2H). ^{13}C NMR (150 MHz, $CDCl_3$): δ 174.5, 168.1, 164.8 (d, $J_{CF} = 251.8$ Hz), 129.7 (d, $J_{CF} = 9.0$ Hz), 122.4 (d, $J_{CF} = 3.2$ Hz), 116.2 (d, $J_{CF} = 21.9$ Hz), 33.3.

3.4.6. 3-(3-Chlorophenyl)-5-(chloromethyl)-1,2,4-oxadiazole 3f. To a solution of **2f** (16.4 g, 0.096 mol) in anhydrous acetone (300 mL) was added potassium carbonate (13.8 g, 0.100 mol), then chloroacetyl chloride (8.07 mL, 11.3 g, 0.100 mol) was added to the stirred reaction mixture over a period of 3 h. The reaction mixture was stirred an additional 3 h at room temperature, then solvent was evaporated under vacuum to give the product as a white solid. The solid was dissolved in ethyl acetate (500 mL), the organic layer was washed with water (4×100 mL), brine (4×100 mL), dried over Na_2SO_4, and filtered. Solvent was removed in vacuo to give 10.3 g (43%) of the O-acyl adduct as a white crystalline solid, mp 105–108 °C. This O-acyl adduct (10.2 g, 0.041 mol) was added to 150 mL of toluene, then the reaction mixture was heated to reflux and stirred for 6 h. Solvent was removed from the hot reaction mixture under vacuum to give a yellow oil. This oil was chromatographed on silica gel with ethyl acetate/hexane (1:9) to afford 7.47 g of a colorless oil, which crystallized on standing. The product was recrystallized from hexane to give the title compound as a white crystalline solid (6.47 g, 69%), mp 44–46 °C, lit. mp 41–42 °C.[12] ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.08 (m, 1H), 7.97 (d, $J = 7.7$ Hz, 1H), 7.50 (m, 1H), 7.43 (m, 1H), 4.75 (s, 2H). ^{13}C NMR (150 MHz, $CDCl_3$): δ 174.6, 167.9, 135.1, 131.6, 130.3, 127.9, 127.6, 125.6, 33.3.

3.4.7. 3-(2-Chlorophenyl)-5-(chloromethyl)-1,2,4-oxadiazole 3g. To a solution of **2g** (16.5 g, 0.096 mol) in anhydrous acetone (250 mL) was added potassium carbonate (13.8 g, 0.100 mol), then chloroacetyl chloride (8.07 mL, 11.3 g, 0.100 mol) was added to the stirred reaction mixture over a period of 1 h. The reaction mixture was stirred overnight at room temperature, then solvent was removed under reduced pressure to give the product as a white solid. This solid was dissolved in ethyl acetate (400 mL), the organic layer was washed with water (4×100 mL), brine (4×100 mL), dried over Na_2SO_4, and filtered. Solvent was removed in vacuo to give 20.4 g (85%) of the O-acyl adduct as a white solid. This O-acyl adduct (14.1 g, 0.057 mol) was suspended in 200 mL of toluene, then the reaction mixture was heated to reflux and stirred for 6 h. Solvent was removed in vacuo from the hot reaction mixture to give the crude product as a light yellow oil. This oil was chromatographed on silica gel with ethyl acetate/hexane (1:9) to give 12.1 g of the product as a colorless oil, which crystallized on standing, mp 39–40 °C. The solid was recrystallized from hexane/chloroform to give 10.2 g (78%) of the title compound as a white crystalline solid, mp 40–42 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.92 (dd, $J_1 = 7.6$ Hz, $J_2 = 1.8$ Hz, 1H), 7.55 (dd, $J_1 = 7.9$ Hz, $J_2 = 1.3$ Hz, 1H), 7.47–7.37 (m, 2H), 4.78 (s, 2H). Note: these data are consistent with the ^{1}H NMR spectral data provided earlier for this compound.[23] ^{13}C NMR (100 MHz, $CDCl_3$): δ 173.9, 167.7, 133.5, 132.0, 131.8, 131.0, 127.0, 125.5, 33.3.

3.4.8. 3-(3,4-Dichlorophenyl)-5-(bromomethyl)-1,2,4-oxadiazole 3h. To a solution of **2h** (16.1 g, 0.078 mol) dissolved in 500 mL of dry acetone was added potassium carbonate (12.3 g, 0.078 mol). Bromoacetyl bromide (15.8 g, 0.078 mol) dissolved in 20 mL of dry acetone was added dropwise to the stirred reaction mixture over a period of 3 h, then the reaction mixture was allowed to stir for an additional 2 h. Solvent was removed in vacuo to give the crude product as a slightly yellow solid. The solid was dissolved in ethyl acetate (500 mL), the organic layer was washed with water (4×100 mL), dried with Na_2SO_4, then filtered. Solvent was evaporated to give 21.5 g of the crude product. Two recrystallizations of the adduct from ethanol afforded 8.89 g (35%) of the O-acyl adduct as a white crystalline solid, mp 129–130 °C. This O-acyl adduct (7.87 g, 0.024 mol) was added to 300 mL of toluene, then the reaction mixture was heated to reflux and stirred for 5 h. Removal of the solvent in vacuo gave a light yellow oil, which solidified on

standing. The crude product was dissolved in a minimum volume of ethyl acetate and chromatographed on silica gel with ether/hexane (1:9). Elution of the column gave 5.75 g (78%) of the product as a white crystalline solid, mp 54–55 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.16 (d, $J = 1.9$ Hz, 1H), 7.90 (dd, $J_1 = 8.4$ Hz, $J_2 = 2.0$ Hz, 1H), 7.55 (d, $J = 8.4$ Hz, 1H), 4.55 (s, 2H). ^{13}C NMR (150 MHz, $CDCl_3$): δ 175.5, 167.7, 136.3, 133.9, 131.5, 129.7, 126.9, 126.5, 16.7.

3.5. Preparation of 3-aryl-5-thio-cyanatomethyl-1,2,4-oxadiazoles

3.5.1. 3-Phenyl-5-(thiocyanatomethyl)-1,2,4-oxadiazole 4a. To a solution of **3a** (3.0 g, 0.013 mol) in 40 mL of dry DMF was added ammonium thiocyanate (2.2 g, 0.03 mol). The reaction mixture was heated and stirred at 70–90 °C for 1 h, then the solution was cooled and ice/water (40 mL) was added with stirring to produce a yellow precipitate. Filtration of the solid followed by washing with water (200 mL) afforded 1.9 g of a yellow powder. The product was chromatographed on silica gel with ethyl acetate/hexane (1:4) as eluent to give the product as a white solid. This solid was recrystallized from hexane/chloroform to give 1.06 g (39%) of the title compound as a white crystalline solid, mp 77–79 °C, lit. 75–77 °C.[13] The analytical sample was obtained by sublimation of the recrystallized solid at 65–70 °C (0.50–1.0 Torr). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.10–8.08 (m, 2H), 7.56–7.47 (m, 3H), 4.40 (s, 2H); ^{13}C NMR (150 MHz, $CDCl_3$): δ 172.8, 169.0, 131.7, 129.0, 127.5, 125.8, 109.4, 27.4. Anal. ($C_{10}H_7N_3OS$) C, H, N.

3.5.2. 3-(4-Chlorophenyl)-5-(thiocyanatomethyl)-1,2,4-oxadiazole 4b. To a solution of **3b** (7.04 g, 0.026 mol) dissolved in 30 mL of dry DMF was added ammonium thiocyanate (4.57 g, 0.06 mol) in 20 mL of dry DMF. The reaction mixture was heated and stirred at 70–90 °C for 75 min, then ice/water (50 mL) was added to the cooled reaction mixture. A yellow precipitate formed, which was filtered and washed with water (100 mL). This yellow precipitate was dissolved in ethyl acetate (500 mL), the organic layer was extracted with distilled water (2×200 mL) and brine (2×200 mL), dried over Na_2SO_4, then filtered. Removal of the solvent in vacuo gave 5.86 g of crude oxadiazole as a yellow crystalline solid. The product was dissolved in a minimum amount of ethyl acetate and chromatographed on silica gel with ethyl acetate/hexane (1:9) to give 5.4 g (83%) of the title compound as light yellow crystals, mp 100–102 °C. The analytical sample was obtained by sublimation of the light yellow solid at 85–90 °C (1 Torr) to give the compound as a white crystalline solid, mp unchanged. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.03 (d, $J = 8.6$ Hz, 2H), 7.48 (d, $J = 8.6$ Hz, 2H), 4.39 (s, 2H); ^{13}C NMR (150 MHz, $CDCl_3$): δ 173.1, 168.3, 138.0, 129.4, 128.9, 124.4, 109.4, 27.3. Anal. ($C_{10}H_6ClN_3OS$) C, H, N.

3.5.3. 3-(4-Methoxyphenyl)-5-(thiocyanatomethyl)-1,2,4-oxadiazole 4c. To a solution of **3c** (3.0 g, 0.013 mol) in 30 mL of dry DMF was added ammonium thiocyanate (2.6 g, 0.030 mol). The solution was heated and stirred at 60–90 °C for 30 min. Ice/water (80 mL) was added to the cooled reaction mixture followed by stirring for 20 min until precipitation of the product was complete. The yellow precipitate was filtered, washed with water (3×80 mL), then chromatographed on silica gel using ethyl acetate/hexane (1:3) as eluent to give 1.8 g (56%) of the title compound as a light yellow crystalline solid, mp 90–92 °C. An analytical sample was obtained by sublimation of the product at 80–86 °C (0.5–0.75 Torr) to give a white crystalline solid, mp unchanged. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.02 (d, $J = 8.9$ Hz, 2H), 7.00 (d, $J = 8.9$ Hz, 2H), 4.37 (s, 2H), 3.87 (s, 3H). ^{13}C NMR (150 MHz, $CDCl_3$): δ 172.5, 168.8, 162.3, 129.2, 118.3, 114.4, 109.5, 55.4, 27.4. Anal. ($C_{11}H_9N_3O_2S$) C, H, N.

3.5.4. 3-(4-Methylphenyl)-5-(thiocyanatomethyl)-1,2,4-oxadiazole 4d. To a solution of **3d** (3.00 g, 0.014 mol) in 25 mL of dry DMF was added ammonium thiocyanate (2.26 g, 0.030 mol). The solution was heated and stirred at 70–95 °C for 30 min. Ice/water (60 mL) was added to the cooled reaction mixture followed by stirring for 30 min until precipitation of the product was complete. The yellow solid that was formed was filtered, washed with water (3×50 mL), and dissolved in ethyl acetate (200 mL). This organic layer was washed with water (1×50 mL), brine (3×50 mL), dried over Na_2SO_4, then solvent was evaporated to give 3.08 g of a light yellow oil, which crystallized on standing. Chromatography on silica gel using ethyl acetate/hexane (1:3) as eluent gave 3.00 g of product as a light yellow crystalline solid. Recrystallization of the product from hexane/ethyl acetate afforded 1.82 g (56%) of the compound as a white crystalline solid, mp 96–98 °C. The analytical sample was obtained by sublimation of the product at 80–85 °C (0.75 Torr). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.98 (d, $J = 7.9$ Hz, 2H), 7.31 (d, $J = 7.9$ Hz, 2H), 4.39 (s, 2H), 2.43 (s, 3H). ^{13}C NMR (150 MHz, $CDCl_3$): δ 172.7, 169.1, 142.2, 129.7, 127.5, 123.1, 109.4, 27.5, 21.6. Anal. ($C_{11}H_9N_3OS$) C, H, N.

3.5.5. 3-(4-Fluorophenyl)-5-(thiocyanatomethyl)-1,2,4-oxadiazole 4e. To a solution of **3e** (7.04 g, 0.033 mol) in 30 mL of dry DMF was added ammonium thiocyanate (6.85 g, 0.09 mol). The reaction mixture was stirred and heated with a water bath at 60–95 °C for 30 min. Ice/water (60 mL) was added to the cooled reaction mixture followed by stirring until precipitation of the product was complete. The precipitate was filtered, washed with water (2×100 mL), and the light yellow solid was dissolved in ethyl acetate (500 mL). This organic layer was extracted with water (3×150 mL) and brine (3×150 mL), dried over Na_2SO_4, and filtered. Removal of the solvent afforded 6.50 g of the crude product as a light yellow oil, which crystallized on standing. Gradient elution from a silica gel column (ether/hexane (1:4) to ether/hexane (1:1)) gave 6.38 g of white crystalline solid, which was recrystallized from hexane/chloroform to afford 3.9 g (50%) of the title compound, mp 74–76 °C.

The analytical sample was obtained by sublimation of the recrystallized solid at 60–70 °C (0.75–1 Torr). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.09 (m, 2H), 7.18 (m, 2H), 4.40 (s, 2H); ^{13}C NMR (150 MHz, $CDCl_3$): δ 173.0, 168.3, 164.9 (d, $J_{CF} = 252.5$ Hz), 129.8 (d, $J_{CF} = 8.8$ Hz), 122.1 (d, $J_{CF} = 2.3$ Hz), 116.3 (d, $J_{CF} = 22.1$ Hz), 109.4, 27.4. Anal. ($C_{10}H_6FN_3OS$) C, H, N.

3.5.6. 3-(3-Chlorophenyl)-5-(thiocyanatomethyl)-1,2,4-oxadiazole 4f. To a solution of **3f** (4.0 g, 0.017 mol) in 40 mL of dry DMF was added ammonium thiocyanate (3.88 g, 0.051 mol). This solution was heated and stirred at 60–90 °C for 30 min. To the cooled solution was added ice/water (80 mL), followed by stirring for 15 min to give a light yellow precipitate. The solid was filtered and washed with water (2×100 mL). This solid was dissolved in ethyl acetate (200 mL), then the organic layer was washed with water (3×100 mL), brine (3×100 mL), dried over Na_2SO_4, and the solvent removed in vacuo to give a yellow solid. Chromatography on silica gel employing ethyl acetate/hexane (1:4) as the eluent gave 2.5 g of the product as a pale yellow solid. The product was recrystallized from chloroform to afford 2.35 g (54%) of the title compound as an off-white crystalline solid, mp 113.5–115 °C. The analytical sample was obtained by sublimation of the solid at 90 °C (1 Torr) to give the compound as a white crystalline solid. ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.09 (m, 1H), 7.98 (d, $J = 7.6$ Hz, 1H), 7.52 (m, 1H), 7.45 (dd, $J_1 = J_2 = 7.8$ Hz, 1H), 4.41 (s, 2H); ^{13}C NMR (150 MHz, $CDCl_3$): δ 173.3, 168.1, 135.1, 131.8, 130.4, 127.7, 127.6, 125.7, 109.3, 27.4. Anal. ($C_{10}H_6ClN_3OS$) C, H, N.

3.5.7. 3-(2-Chlorophenyl)-5-(thiocyanatomethyl)-1,2,4-oxadiazole 4g. To a solution of **3g** (4.0 g, 0.017 mol) in 30 mL of dry DMF was added ammonium thiocyanate (3.88 g, 0.051 mol). The reaction mixture was heated and stirred at 60–90 °C for 30 min. Ice/water (80 mL) was added to the cooled reaction mixture followed by stirring for 20 min until precipitation of the product was complete. The yellow solid that was formed was filtered, washed with water (3×50 mL), and dissolved in ethyl acetate (300 mL). This organic layer was washed with water (1×50 mL), brine (3×50 mL), dried over Na_2SO_4, then solvent was evaporated to give the product as a yellow solid. Chromatography on silica gel using ethyl acetate/hexane (1.5:8.5) as eluent gave 3.72 g of product, which was recrystallized from chloroform/hexane to give 3.01 g (70%) of the title compound as a light yellow crystalline solid, mp 86–90 °C. The analytical sample was obtained by sublimation of the light yellow recrystallized compound at 60–80 °C (0.6–0.7 Torr) to give the title compound as a white crystalline compound, mp unchanged. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.96 (dd, $J_1 = 7.6$ Hz, $J_2 = 1.8$ Hz, 1H), 7.56 (dd, $J_1 = 7.9$ Hz, $J_2 = 1.3$ Hz, 1H), 7.49–7.39 (m, 2H), 4.44 (s, 2H). ^{13}C NMR (150 MHz, $CDCl_3$): δ 172.5, 167.9, 133.5, 132.2, 131.8, 131.1, 127.0, 125.2, 109.4, 27.3. Anal. ($C_{10}H_6ClN_3OS$) C, H, N.

3.5.8. 3-(3,4-Dichlorophenyl)-5-(thiocyanatomethyl)-1,2,4-oxadiazole 4h. To a solution of **3h** (3.17 g, 0.01 mol) in 50 mL of dry DMF was added ammonium thiocyanate (2.6 g, 0.03 mol). The reaction mixture was heated and stirred at 75–95 °C for 1 h, then cooled. Ice/water (50 mL) was added with stirring to the reaction mixture to precipitate the product, then the yellow solid was filtered, washed with water, and dissolved in ethyl acetate (500 mL). The organic layer was washed with water (3×100 mL), brine (2×100 mL), and dried (Na_2SO_4). Solvent was removed in vacuo to give the crude product as an orange-yellow solid. Gradient elution of the product from a silica gel column using chloroform/hexane (1:4 to 1:1) afforded 1.87 g of product. Recrystallization from chloroform-hexane gave 1.44 g (50%) of the title compound as a light yellow solid, mp 106–108 °C. Sublimation of the solid at 90–95 °C (0.50 Torr) gave the analytical sample as a white crystalline solid, mp 108–109 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.20 (d, $J = 2.0$ Hz, 1H), 7.94 (dd, $J_1 = 8.4$ Hz, $J_2 = 2.0$ Hz, 1H), 7.59 (d, $J = 8.4$ Hz, 1H), 4.40 (s, 2H). ^{13}C NMR (150 MHz, $CDCl_3$): δ 173.4, 167.5, 136.2, 133.6, 131.2, 129.4, 126.6, 125.8, 109.2, 27.3. Anal. ($C_{10}H_5Cl_2N_3OS$) C, H, N.

3.6. Preparation of 3-aryl-5-alkyl-1,2,4-oxadiazoles 5a–f

These compounds were prepared from benzamidoximes **2a–c** and acetyl or isobutyryl chloride in pyridine according to the general procedure of Chiou and Shine.[15] *Representative procedure for the preparation of 3-(4-methoxyphenyl)-5-methyl-1,2,4-oxadiazole* **5c**. To a three-necked flask equipped with an addition funnel and a condenser was added **2c** (3.04 g, 0.018 mol) and pyridine (4 mL). Acetyl chloride (2.75 g, 0.035 mol) was then added dropwise to this suspension over a period of 15 min, then the reaction mixture was heated to reflux for 45 min. Upon cooling, the mixture solidified. Water (30 mL) was added, at which point the product precipitated. The precipitate was filtered, washed with water, then chromatographed on silica gel using a methylene chloride/hexane (1:4 to 2:3) step gradient as eluent to give 2.90 g (85%) of the desired product as a white crystalline solid, mp 61–63 °C, lit. 58–59 °C.[24] ^{1}H NMR (250 MHz, $CDCl_3$): δ 8.00 (d, $J = 8.9$ Hz, 2H), 6.98 (d, $J = 8.9$ Hz, 2H), 3.87 (s, 3H), 2.64 (s, 3H).

3.6.1. 3-Phenyl-5-methyl-1,2,4-oxadiazole 5a. Melting point 40–41 °C, lit. 39–41 °C.[15] ^{1}H NMR (250 MHz, $CDCl_3$): δ 8.06 (m, 2H), 7.48 (m, 3H), 2.66 (s, 3H).

3.6.2. 3-(4-Chlorophenyl)-5-methyl-1,2,4-oxadiazole 5b. Melting point 103–105 °C, lit. 90–92 °C.[25] ^{1}H NMR (250 MHz, $CDCl_3$): δ 8.01 (d, $J = 8.4$ Hz, 2H), 7.46 (d, $J = 8.4$ Hz, 2H), 2.66 (s, 3H).

3.6.3. 3-Phenyl-5-isopropyl-1,2,4-oxadiazole 5d. Colorless oil,[26] ^{1}H NMR (250 MHz, $CDCl_3$): δ 8.09 (m, 2H), 7.48 (m, 3H), 3.30 (sep, $J = 7.0$ Hz, 1H), 1.47 (d, $J = 7.0$ Hz, 6H).

3.6.4. 3-(4-Chlorophenyl)-5-isopropyl-1,2,4-oxadiazole 5e. Colorless oil,[26] ^{1}H NMR (250 MHz, $CDCl_3$): δ 8.02 (d, $J = 8.4$ Hz, 2H), 7.45 (d, $J = 8.4$ Hz, 2H), 3.28 (sep, $J = 7.0$ Hz, 1H), 1.46 (d, $J = 7.0$ Hz, 6H).

3.6.5. 3-(4-Methoxyphenyl)-5-isopropyl-1,2,4-oxadiazole 5f. Colorless oil,[26] ^{1}H NMR (250 MHz, $CDCl_3$): δ 8.01 (d, $J = 8.4$ Hz, 2H), 6.98 (d, $J = 8.4$ Hz, 2H), 3.87 (s, 3H), 3.27 (sep, $J = 7.1$ Hz, 1H), 1.45 (d, $J = 7.1$ Hz, 6H).

3.7. In vitro assays

The activity of the test compounds against *L. donovani* axenic amastigotes (WHO designation MHOM/SD/62/1S-$CL2_D$) was measured as described previously.[5,27] Assessment of the antiproliferative activity of compounds against bloodstream-form *Trypanosoma brucei brucei* (MITat 1.2, variant 221) and J774 murine macrophages was also carried out as outlined earlier.[5] The toxicity of compounds to PC3 cells and Vero cells was measured in the same manner as for the macrophages, except that the medium differed in each case. For the PC3 cells, the medium was F12K (Kaighn's modification, from Gibco) supplemented with 50 units/mL penicillin, 50 μg/mL streptomycin, and 10% fetal bovine serum, while Vero cells were grown in Eagle's Minimal Essential Medium with Earle's Balanced Salt Solution (from ATCC), which includes 2 mM glutamine, non-essential amino acids and is supplemented with 50 units/mL penicillin, 50 μg/mL streptomycin, and 10% fetal bovine serum. Selected compounds were tested for their activity against *Leishmania*-infected J774 macrophages essentially as described earlier[28] with some modifications. Briefly, macrophages were mixed with a late log phase culture of *L. mexicana* (WHO designation: MNYC/BCZ/62/M379) promastigotes to yield a solution containing 5×10^5 macrophages/mL and 37.5×10^5 promastigotes/mL in DMEM supplemented with 2 mM L-glutamine, 50 units/mL penicillin, 50 μg/mL streptomycin, and 10% heat-inactivated fetal calf serum. The macrophage–parasite mixture was then pipetted into 96-well flat-bottom plates at 200 μL/well. Infection and attachment of the macrophages was allowed to occur over a period of 24 h at 33 °C in a humidified 5% CO_2 incubator. Wells were washed three times with Hank's Balanced Salt Solution to remove extracellular parasites, then serial dilutions of drugs in supplemented DMEM were added to each well. The plate was returned to the CO_2 incubator for an additional 72 h incubation at 33 °C. Macrophages in each well were subsequently detached by pipetting and scraping with the tip of a microliter pipettor. A portion of the cell suspension (75 μL) was transferred to a cytospin funnel and the cells were centrifuged onto microscope slides at 800 rpm for 5 min using a Cytospin centrifuge (Shandon). The slides were allowed to air dry, then were fixed in methanol for 5 s. After evaporation of the methanol, the slides were stained with 5% Giemsa stain (Fisher) in phosphate buffer (3.1 mM potassium phosphate dibasic, 8.3 mM sodium phosphate monobasic) for 45 min. After thorough washing in flowing tap water, the slides were allowed to air dry before being viewed by oil immersion microscopy to determine the percentage of infected cells. For each in vitro determination, experiments were performed at least twice, with the mean ± standard deviation given in each case.

3.8. In vivo antileishmanial assays

The in vivo efficacy of compounds against *L. donovani* (strain MHOM/ET/67/82/L82) was measured according to general procedures outlined previously.[18] In these experiments, female BALB/c mice were infected intravenously, via the lateral tail vein, with 1.5×10^7 *L. donovani* amastigotes freshly isolated from the spleen of an infected hamster. Infected mice were then randomly sorted into groups of five. Treatment groups received the test or standard compound subcutaneously (s.c.), intravenously (i.v.), or orally (p.o.) once per day for 5 days on days 7–11 of infection. In addition, one group of five mice was given the drug vehicle only. Three days after the completion of treatment, the mice were killed and their livers were removed and weighed. Smears were then prepared, methanol fixed, and Giemsa stained. The activity of the compounds was determined by comparing the number of amastigotes per 500 liver cells times the tissue weight in animals from treated and untreated groups.

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