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Antikinetoplastid antimitotic activity and metabolic stability of dinitroaniline sulfonamides and benzamides

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Abstract—N^1-Phenyl-3,5-dinitro-N^4,N^4-di-*n*-propylsulfanilamide (**1**) and N^1-phenyl-3,5-dinitro-N^4,N^4-di-*n*-butylsulfanilamide (**2**) show potent in vitro antimitotic activity against kinetoplastid parasites but display poor in vivo activity. Seventeen new dinitroaniline sulfonamide and eleven new benzamide analogs of these leads are reported here. Nine of the sulfonamides display in vitro IC_{50} values under 500 nM against African trypanosomes, and the most active antikinetoplastid compounds also inhibit the in vitro assembly of purified leishmanial tubulin with potencies similar to that of **2**. While several of the potent compounds are rapidly degraded by rat liver S9 fractions in vitro, N^1-(3-hydroxy)phenyl-3,5-dinitro-N^4,N^4-di-*n*-butylsulfanilamide (**21**) displays an IC_{50} value of 260 nM against African trypanosomes in vitro and is more stable than **2** in the in vitro metabolism assay.

1. Introduction

Leishmaniasis, African trypanosomiasis, and Chagas disease are vector-borne diseases affecting millions in the developing world that are caused by parasites of the order kinetoplastida. The clinical manifestations of leishmaniasis, which range from limited, self-healing cutaneous lesions to fatal visceral disease, depend on the identity of the *Leishmania* species responsible for infection. African trypanosomiasis is always fatal in the absence of treatment; infection with *Trypanosoma brucei rhodesiense* results in death within a few months, while *Trypanosoma brucei gambiense* infections follow a longer course. The parasite responsible for Chagas disease, *Trypanosoma cruzi*, causes severe chronic disease affecting the heart, esophagus, and colon. Further information regarding the geographical distribution, prevalence, transmission, and symptoms of these diseases is available online from the World Health Organization (see <http://www.who.int/leishmaniasis/disease_epidemiology/en/index.html/>, <http://www.who.int/mediacentre/factsheets/fs259/en//>, and <http://www.who.int/ctd/chagas/disease.htm/>). Despite the morbidity and mortality caused by these diseases, their chemotherapy remains unsatisfactory (see Croft et al.[1] for a recent review), underscoring the urgent need for improved antitrypanosomal and antileishmanial drugs.

Dinitroaniline compounds have displayed activity against various parasites,[2–4] including *Leishmania*[5–8] and trypanosomes.[6,9] Tubulin has been proposed to be the antiparasitic target of these molecules.[10–12] We previously showed that both N^1-phenyl-3,5-dinitro-N^4,N^4-di-*n*-propylsulfanilamide (GB-II-5, compound **1**)[13] and N^1-phenyl-3,5-dinitro-N^4,N^4-di-*n*-butylsulfanilamide (GB-II-150, compound **2**)[14] possess selective antimicrotubule activity against *Leishmania* and African trypanosomes in vitro. A decrease in alkyl chain length at N^4 and substitution

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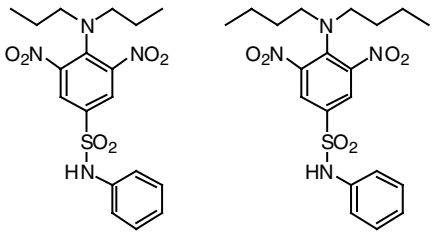
of N^1-alkyl chains for the N^1 aromatic ring diminished antiparasitic activity, as did large substituents on the N^1 aromatic ring.[14] Despite their in vitro activity, **1** and **2** showed no significant in vivo activity when administered ip at 20 mg/kg/day for four days in a murine model of African trypanosomiasis.[14]



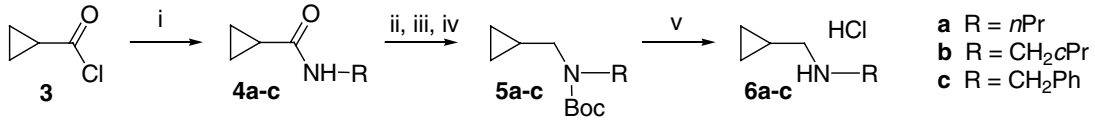
1 **2**

To provide a rationale for the lack of in vivo antitrypanosomal efficacy of these compounds, pharmacokinetic and metabolism studies were conducted with **2**.[15] As expected, the compound was extensively metabolized in vitro and in vivo, with the major products resulting from N^1 ring oxidation, N^4 alkane oxidation, and N^4 oxidation. Based on the results of these studies, we have prepared analogs of **1** and **2** in an effort to achieve greater metabolic stability while maintaining selective antiparasitic activity. The synthesis and biological evaluation of these compounds is reported here.

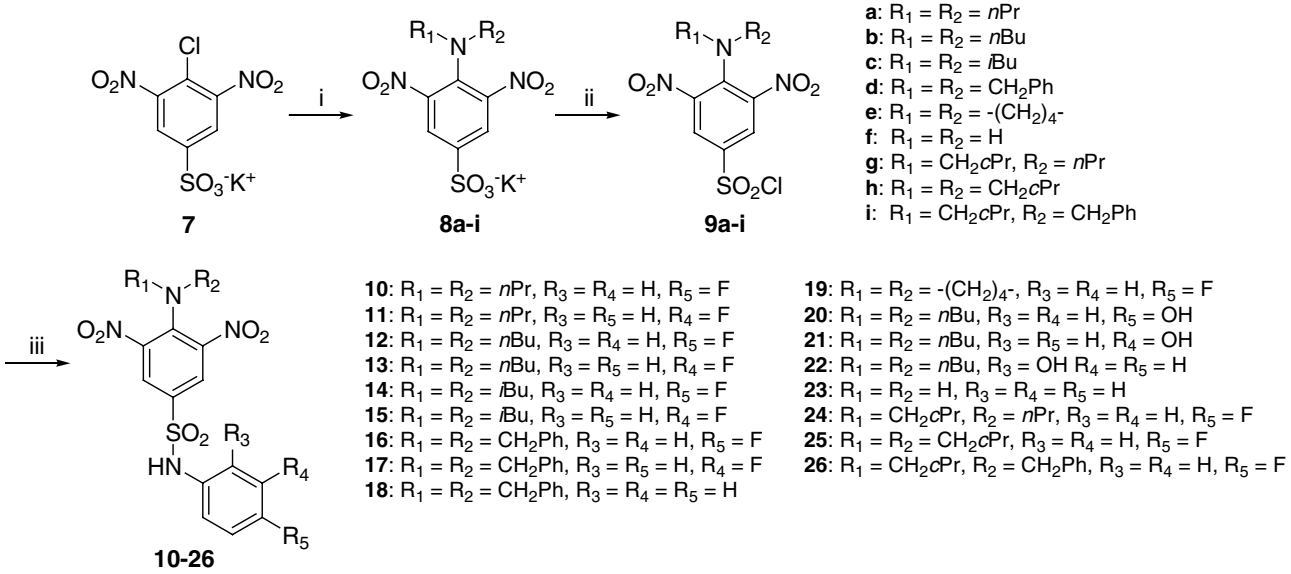
2. Results and discussion

2.1. Chemistry

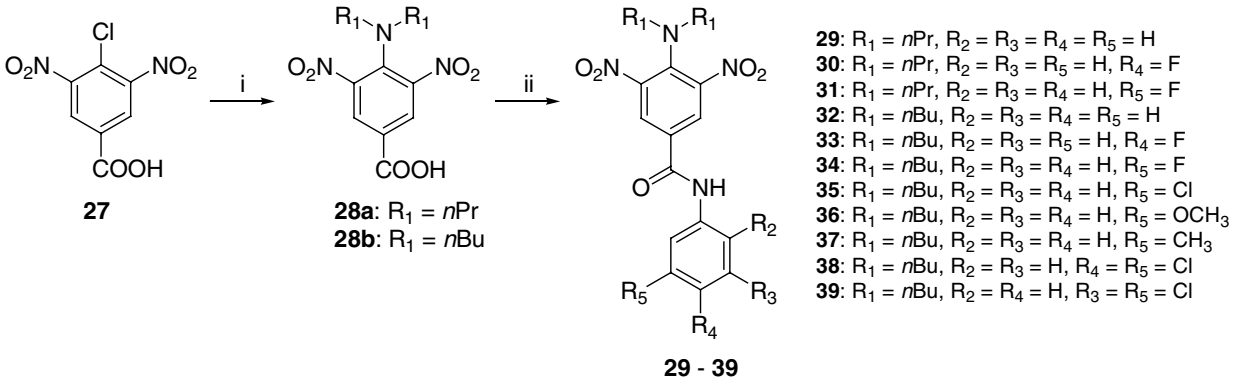
Reaction of primary amines with cyclopropanecarbonylchloride (**3**) followed by amide reduction, methanolysis, trapping with di-*tert*-butyl dicarbonate, and deprotection with HCl (Scheme 1) as described by Dubowchik et al.[16] provided secondary amine hydrochlorides **6a–c** containing a cyclopropylmethyl group. Dinitroaniline sulfonamides were synthesized as outlined in Scheme 2 by the general methods reported earlier.[14] Reaction between 3,5-dinitro-4-chloro-benzenesulfonate **7**[17] and the desired secondary amines afforded intermediates **8a–i** in high yield, which were converted to sulfonyl chlorides **9a–i** by treatment with PCl_5 in dichloromethane as solvent. The target sulfonamides **10–26** were prepared from **9a–i** by reaction of these sulfonyl chlorides with aromatic amines in pyridine at 40 °C. Dinitroaniline benzamides were prepared as shown in Scheme 3. Commercially available 4-chloro-3,5-dinitrobenzoic acid **27** was reacted with either dipropylamine or dibutylamine (15 equiv) in methanol at reflux to provide benzoic acids **28a** and **28b** in high yield. Benzamides **29–39** were synthesized by coupling **28a** or **28b** with 1.5 equiv of the desired aromatic amines in dichloromethane at room temperature in the presence of DCC and catalytic DMAP.



Scheme 1. Reagents and conditions: (i) RNH_2, Et_3N, CH_2Cl_2, 0 °C to rt; (ii) BH_3–THF (3 equiv), reflux, 14 h; (iii) MeOH, reflux, 2 h; (iv) Boc_2O (1.4 equiv), CH_2Cl_2, rt, 14 h; (v) 4 M HCl/dioxane (1.2 equiv), CH_2Cl_2, rt, 14 h.



Scheme 2. Reagents and conditions: (i) secondary amine/reflux, secondary amine/MeOH/reflux, or NH_3/H_2O/reflux; (ii) PCl_5/CH_2Cl_2; (iii) NH_2Ar/pyridine/40 °C.



Scheme 3. Reagents and conditions: (i) dipropylamine/MeOH/reflux or dibutylamine/MeOH/reflux; (ii) NH_2Ar/DCC/DMAP/CH_2Cl_2.

2.2. Biological evaluation

Since earlier studies indicated that the N^4-dialkyl groups and the N^1-phenyl ring of **2** were extensively metabolized,[15] synthetic modifications to lead compounds **1** and **2** aiming to increase metabolic stability focused on these two regions of the molecules. Substitution with a fluorine atom was attempted on the N^1-phenyl ring because the presence of larger groups led to a significant loss of activity.[14] Potent antikinetoplastid activity was maintained when a fluorine atom was placed either *para* or *meta* to the sulfonamide group in both N^4,N^4-di-*n*-propyl compounds **10** and **11** and N^4,N^4-di-*n*-butyl compounds **12** and **13**, with the *para*-substituted compounds being more active against *T. b. rhodesiense* in vitro (Table 1). Compounds **10** and **12** also maintained selective activity for leishmanial tubulin (Table 2). However, substantial losses in antiparasitic activity were observed when the N^4 position was disubstituted with branched chains (compounds **14** and **15**) or benzyl groups (compounds **16**–**18**), particularly against African trypanosomes. For example, N^4,N^4-diisobutyl analog **14** is 19- and 11-fold less potent against *T. b. rhodesiense* than the corresponding N^4,N^4-di-*n*-propyl compound **10** and N^4,N^4-di-*n*-butyl analog **12**, respectively. Interestingly, such substitutions did not lead to a decrease in toxicity to mammalian L6 cells, suggesting that this class of compounds exerts antiparasitic and toxic effects through different mechanisms. While these bulky substitutions at N^4 in **14**–**18** led to a loss of antikinetoplastid activity, so did the presence of a pyrrolidine ring (compound **19**), and the absence of an N^4 dialkyl group (compound **23**) led to decreased antileishmanial activity compared to **1**, **2**, and **10**–**13**.

Compounds **24** and **25**, which contained either one or two cyclopropylmethyl groups at N^4, possessed activity against *Leishmania donovani* and *T. b. rhodesiense* that was comparable to that of **1**, **2**, and **10**–**13**. Surprisingly, the presence of these cyclopropyl groups at N^4 led to a decrease in selectivity for parasite tubulin. While **24** and **25** retained strong activity in blocking the assembly of purified leishmanial tubulin, these compounds were only 2.4- and 2.8-fold more potent against parasite tubulin assembly compared to porcine tubulin assembly (Table 2). Although the observation that **24** and **25** possess activity against mammalian tubulin was unanticipated, it is likely that subtle distinctions between tubulins account for the differences in susceptibilities between the leishmanial and mammalian proteins in binding to dinitroanilines. In the protozoan parasite *Toxoplasma gondii*, organisms resistant to the dinitroaniline herbicide oryzalin generated by chemical mutagenesis possessed numerous point mutations in α-tubulin.[10] The majority of these point mutations occurred within the core of α-tubulin and not at the proposed binding site for dinitroanilines determined by docking simulations.[10] Also, the proposed binding site residues identified in these docking studies are not specific to protozoa, suggesting that minor differences between mammalian and protozoal tubulin account for dinitroaniline specificity. In addition, our mammalian tubulin assay, in which the protein does not assemble as robustly as does the parasite protein, may be more sensitive to assembly inhibition than our leishmanial tubulin assay, in which the protein assembles rapidly.[13] Supporting this idea, Hamel has shown that differing conditions employed in tubulin assembly assays have a profound effect on experimentally determined IC_{50} values.[18]

Compound **26**, which possessed the mixed benzyl-cyclopropylmethyl substitution pattern at N^4, showed antileishmanial activity that was slightly lower than **1**, **2**, and **10**–**13**, but was as potent as **2** against *T. b. rhodesiense* and also displayed selective activity for leishmanial tubulin. The activity of **26** against leishmanial tubulin demonstrates that a single substituent at N^4 with added bulk compared to an alkyl chain is permitted for binding to this parasite protein. Considering the poor activity of dibenzyl compounds **16**–**18** against *T. b. rhodesiense*, the potency of **26** against these organisms is surprising. Because large quantities of assembly-competent tubulin from *T. brucei* subspecies are not currently available, an assessment of the cellular antimitotic effects of several of these compounds on African trypanosomes at their IC_{50} concentrations will be required in order to provide further evidence that these new dinitroaniline sulfonamides exert their trypanocidal effects through a tubulin mechanism.

The activity of N^1-aryl-3,5-dinitrosulfanilamides against intracellular *T. cruzi* is presented for the first time in

Table 1. Activities of dinitroanilines (μM) against parasites and mammalian cells[a]

Compound	IC_{50} versus *L. donovani* axenic amastigotes[a]	IC_{50} versus *T. b. rhodesiense*[b]	IC_{50} versus *T. cruzi*[b]	IC_{50} versus L6 cells[b]	IC_{50} versus Vero cells[a]
1	5.8	0.18	1.9	17	26
2	3.5	0.12	0.59	12	19
10	5.7	0.17	7.5	20	6.3
11	4.9	0.48	7.0	22	9.3
12	5.1	0.28	3.0	10	2.9
13	4.5	1.4	1.8	6.2	3.3
14	9.3	3.2	9.6	11	NT[d]
15	10	2.6	11	14	NT
16	12	19	10	18	NT
17	6.9	19	8.9	15	NT
18	14	14.5	8.6	21	NT
19	73	9.9	51	70	NT
20	15	21	2.6	12	NT
21	2.8	0.26	1.3	13	24
22	8.6	22	9.6	13	NT
23	19	NT	NT	NT	NT
24	4.5	0.37	7.3	14	NT
25	3.7	0.31	11	15	NT
26	8.4	0.13	13	20	NT
29	>100	29	21	>100	NT
30	>100	99	>50	64	NT
31	22	30	12	17	NT
32	>100	24	6.5	23	NT
33	>100	33	>50	>50	NT
34	17	21	5.1	16	NT
35	22	33	5.1	14	NT
36	20	23	3.8	41	NT
37	56	15	3.5	20	NT
38	22	22	7.8	6.0	NT
39	39	34	23	14	NT
Pentamidine	2.4	NT	NT	4.2[c]	>100
Melarsoprol	NT	0.015	NT	NT	NT
Benznidazole	NT	NT	3.4	NT	NT
Podophyllotoxin	NT	NT	NT	0.01	NT

[a] Activities represent the mean of three independent experiments; the standard error is less than 30% of the mean in all cases.
[b] Activities represent the mean of at least two independent experiments; IC_{50} values used to calculate the average for a given compound are within a factor of two.
[c] Value obtained from Ref. 26.
[d] NT, not tested.

Table 2. Antimicrotubule activity of selected compounds[a]

Compound	IC_{50} (μM) versus *L. tarentolae* tubulin	IC_{50} (μM) versus porcine tubulin
2	6.5 ± 0.3	>40
10	5.3 ± 0.3[b]	>40
12	5.1 ± 1.4	>40
20	17 ± 3[b]	9.7 ± 1.0
21	5.6 ± 0.7	8.2 ± 0.2
24	4.5 ± 0.2	11 ± 1
25	3.9 ± 1.1	11 ± 4
26	5.4 ± 0.8	>40
29	>40	>40

[a] Unless otherwise noted, values represent means ± range of two duplicate experiments.
[b] Values represent the mean ± standard error of at least three duplicate experiments.

Table 1. Compounds **2**, **12**, and **21** are among the most active of these agents against the American trypanosome, and all of these compounds also display strong activity against purified leishmanial tubulin in vitro. However, compounds **10**, **24**, and **25** also show potent activity against leishmanial tubulin but are less active against intracellular *T. cruzi* in vitro. While subtle differences in susceptibility between leishmanial tubulin and *T. cruzi* tubulin could help to account for these differences, the presence of a host cell membrane may also have a profound influence on the accumulation of the compounds within the American trypanosome, interfering with correlations between antitubulin activity and potency against intracellular *T. cruzi*.

Compounds **20**–**23** were prepared as potential metabolites of **2**. Through comparison of HPLC retention times and fragmentation patterns with synthetic standards, compounds **20**, **22**, and **23** were identified as metabolites of this lead compound.[15] Interestingly, the three metabolites in general show low antikinetoplastid activity, while **21** displays in vitro antileishmanial and antitrypanosomal activity comparable to that of **1** and **2**. The in vitro activity of **21** against kinetoplastid parasites and leishmanial tubulin assembly suggests that the *meta*

hydroxyl group present in this molecule may participate in hydrogen bonding with the tubulin target, compensating for the otherwise unfavorable steric interactions that were observed in most other N^1-phenyl substituted compounds in this series.[14] As with **24** and **25**, a decrease in selectivity for parasite tubulin was also observed with compounds **20** and **21**, and **20** showed higher activity against porcine tubulin than against leishmanial tubulin. Despite this, mitotic arrest was not observed in MCF-7 breast cancer cells incubated for 24 h with 3, 10, and 30 μM **20** (Dan Sackett, data not shown). We hypothesize that other cellular targets are affected by **20** in mammalian cells prior to tubulin. It is worth noting that compounds **21**, **24**, and **25** maintain selectivity for kinetoplastid parasites over mammalian cells, particularly against African trypanosomes.

We also prepared dinitroaniline benzamides **29**–**39** to examine the effect of changing the linker between the two aromatic rings in this series of molecules. In general, substitution of the amide for the sulfonamide group leads to significant losses in antikinetoplastid activity. For example, benzamide **29** shows IC_{50} values of >100 and 29 μM versus *L. donovani* and *T. b. rhodesiense*, respectively, while the corresponding sulfonamide **1** displays IC_{50} values of 5.8 and 0.18 μM against these parasites. While amides that contained either an unsubstituted aniline ring or that possessed a *para*-fluoro atom on this ring were inactive, *meta*-fluorinated compounds **31** and **34** showed moderate activity against *L. donovani* and *T. cruzi*, encouraging us to prepare *meta*-substituted compounds **35**–**39**. Although **36** and **37** showed reasonable activity against *T. cruzi*, sulfonamides **1**, **2**, **13**, and **21** remained more potent against this parasite. Thus the dinitroaniline benzamides were not investigated further.

Given the extensive metabolism of **2**,[15] a major goal of this work was to prepare active antimitotic antikinetoplastid agents with improved metabolic stability. We thus examined the rate at which selected compounds were converted to other molecules upon incubation with the S9 fraction of rat liver microsomes (Table 3). The presence of an N^4-dibenzyl substitution stabilized compounds **16** and **18** to metabolism compared to **2**, with **16** and **18** displaying 2.2- and 2.1-fold greater in vitro stability compared with **2** when incubated with the S9 fraction for 10 min. In addition, the pyrrolidine-containing compound **19** also displayed greater in vitro stability than **2**. N^4-Dibutyl, N^4-isobutyl, and N^4-cyclopropylmethyl containing compounds **12**, **14**, and **25**, which all possess a *para*-fluoro group on the N^1 ring, were less stable than compound **2**. Taken together, these data suggest that sufficiently bulky substituents at the N^4 position stabilize the compounds to metabolism compared with **2**, while the addition of a *para*-fluoro group on the N^1 ring does not prevent degradation, perhaps due to the greater influence of the N^4 groups on metabolic oxidation. The role played by the N^4 dialkyl groups in rapid metabolism is further highlighted by the stability of **23**, which contains an unsubstituted anilino nitrogen atom. Unfortunately, **16**, **18**, and **23** display low activity against kinetoplastid parasites. Interestingly, **26** shows the lowest metabolic stability, suggesting that dibenzyl groups are required at N^4 to introduce sufficient steric bulk to slow in vitro metabolism. Compounds **20** and **21**, which possess *para* and *meta* hydroxyl groups on the N^1 phenyl ring, respectively, display greater metabolic stability compared to **2**. These data suggest that introduction of polar functional groups may increase metabolic stability in this series of molecules. Compound **21** displays similar antiparasitic and antitubulin activity compared to **2** and retains its selectivity for kinetoplastid parasites compared to mammalian cell lines, particularly for African trypanosomes.

Table 3. In vitro metabolism of selected compounds in the presence of rat liver S9 enzymes

Compound	Percent remaining at 10 min[a]
2	31 ± 3
12	20 ± 1
14	21 ± 3
16	69 ± 3
18	64 ± 4
19	49 ± 9
20	48 ± 1
21	45 ± 5
23	115 ± 12
25	6 ± 0
26	1 ± 0

[a] Values represent means ± standard error of two triplicate experiments ($N = 6$).

3. Conclusion

Based on in vitro antikinetoplastid activity, selectivity, and metabolic stability, compound **21** emerges as a candidate for further evaluation against kinetoplastid parasites. This compound also provides a possible starting point for the synthesis of further analogs with improved metabolic characteristics and for the preparation of dinitroaniline sulfanilamide prodrug candidates against kinetoplastid parasites.

Several new dinitroaniline sulfonamides have been identified that possess selective activity against leishmanial tubulin, while others also display activity against the corresponding mammalian protein. Such observations should not dampen enthusiasm for the investigation of tubulin as an antiprotozoal drug target. For example, while the dihydrofolate reductase (DHFR) inhibitor methotrexate is highly toxic to mammalian cells and bacteria alike, the success of the DHFR inhibitor trimethoprim as antibacterial agent cannot be disputed. While the structural basis of the preference of trimethoprim for prokaryotic dihydrofolate reductase was not immediately appreciated,[19] further study of the ligand binding properties of prokaryotic and eukaryotic DHFR shed light on the basis of antimicrobial selectivity and led to the identification of new, selective ligands.[20,21] Future studies focused on providing a model for the interaction between kinetoplastid tubulin and the dinitroanilines could be useful in identifying compounds with increased potency and selectivity for the parasite protein. Given

the vital cellular role of tubulin, the potency of dinitroaniline sulfonamides against kinetoplastid parasites, and their selectivity profile against leishmanial and mammalian tubulins, further exploration of this target protein in protozoans is warranted.

4. Experimental

4.1. 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 or 300 MHz for ^{1}H and 62.5 or 75 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 unless otherwise noted. Thin layer chromatography was conducted on precoated TLC plates from E. Merck. Compounds **1** and **2** were prepared as described previously.[14]

4.2. General procedure for the synthesis of secondary amine hydrochlorides 6a–c[16]

To a solution of the primary amine or primary amine hydrochloride (1 equiv) in dry CH_2Cl_2 at 0 °C, triethylamine (1.5–3 equiv) was added followed by cyclopropanecarbonyl chloride (1 equiv). After 10 min, the ice bath was removed and stirring was continued at rt overnight. Dichloromethane was removed in vacuo and the residue was partitioned between EtOAc and saturated $NaHCO_3$. The aqueous layer was further extracted with EtOAc, the combined organic layers were washed with H_2O and brine and concentrated in vacuo. This residue was dried under high vacuum, then treated with 1 M BH_3 in THF (3 equiv). The mixture was heated at reflux for 14 h and then cooled to 0 °C. MeOH was carefully added and the solution was heated to reflux for 5 h. Upon recooling to 0 °C, a solution of di-*tert*-butyl dicarbonate (1.4 equiv) in CH_2Cl_2 was added, the resulting mixture was stirred at rt overnight, solvent was removed in vacuo, then the residue was partitioned between EtOAc and water. The organic phase was washed with water, brine, dried over $MgSO_4$, and concentrated in vacuo to give the crude Boc protected amine. This protected amine was dissolved in CH_2Cl_2 and treated carefully with 4 M HCl in dioxane (1.2 equiv). The mixture was stirred at rt overnight and concentrated in vacuo. The resulting white solid was triturated with ether, collected by filtration, washed with ether, and dried in vacuo.

4.2.1. *N*-(Cyclopropylmethyl)-*N*-propylamine hydrochloride (6a). Prepared from cyclopropanecarbonyl chloride (1.5 g, 14.3 mmol) and propylamine (848 mg, 14.3 mmol); yield 651 mg, 31% (after five steps); mp = 276 °C; ^{1}H NMR ($CDCl_3$) δ 0.44–0.50 (m, 2H), 0.68–0.76 (m, 2H), 1.03 (t, 3H, J = 7.6 Hz), 1.23–1.36 (m, 1H), 1.89–2.04 (m, 2H), 2.86–3.02 (m, 4H), 9.56 (br s, 2H).

4.2.2. *N*,*N*-Di(cyclopropylmethyl)amine hydrochloride (6b). Prepared from cyclopropanecarbonyl chloride (972 mg, 9.3 mmol) and cyclopropylmethylamine hydrochloride (1 g, 9.3 mmol); yield 369 mg, 25% (after five steps); mp = 275 °C; ^{1}H NMR ($CDCl_3$) δ 0.46–0.51 (m, 4H), 0.69–0.75 (m, 4H), 1.26–1.39 (m, 2H), 2.92–2.96 (m, 4H), 9.69 (br s, 2H).

4.2.3. *N*-Benzyl-*N*-(cyclopropylmethyl)amine hydrochloride (6c). Reaction of cyclopropanecarbonyl chloride (3.0 g, 28.7 mmol) and benzylamine (3.1 g, 28.7 mmol) afforded cyclopropionyl benzylamide (5.02 g, quantitative). The reduction of cyclopropionyl benzylamide (3 g, 17.1 mmol) afforded **6c**: yield 1.6 g, 47% (after four steps); mp = 184 °C; ^{1}H NMR ($CDCl_3$) δ 0.36–0.41 (m, 2H), 0.58–0.69 (m, 2H), 1.21–1.27 (m, 1H), 2.67–2.73 (m, 2H), 4.09–4.13 (m, 2H), 7.38–7.64 (m, 5H), 9.98 (br s, 2H).

4.2.4. 3,5-Dinitro-N^4,N^4-diisobutylbenzenesulfonate (8c). A suspension of 3,5-dinitro-4-chlorobenzenesulfonate (**7**, 3 g, 9.3 mmol) and diisobutylamine (4.9 mL, 28.1 mmol) in 60 mL methanol was refluxed for 3 h. After 3 h the solvent was evaporated. The product was purified by silica gel column chromatography, eluting with MeOH/EtOAc 1:25; yield 2.74 g, 70%; ^{1}H NMR (DMSO-d_6): δ 0.81 (d, J = 6.6 Hz, 12H), 1.72–1.87 (m, 2H), 2.72 (d, J = 6.9 Hz, 4H), 8.05 (s, 2H). Note: some traces of the dialkylamine salts were often associated with dinitrosulfonates **8a**–**i** as indicated by their ^{1}H NMR spectra which did not interfere with the further reactions.

4.2.5. 3,5-Dinitro-N^4,N^4-dibenzylbenzenesulfonate (8d). A suspension of **7** (2 g, 6.23 mmol) and dibenzylamine (3.6 mL, 18.7 mmol) in 100 mL methanol was refluxed for 3 h. After 3 h the solvent was evaporated. The product was purified by silica gel column chromatography, eluting with MeOH/EtOAc 1:15; yield 2.5 g, 83%; ^{1}H NMR (DMSO-d_6) δ 4.13 (s, 4H), 7.14–7.36 (m, 10H); 8.11 (s, 2H).

4.2.6. 3,5-Dinitro-4-pyrrolidin-1-yl-benzenesulfonate (8e). A suspension of **7** (1 g, 3.12 mmol) and pyrrolidine (0.5 mL, 6.1 mmol) in 20 mL methanol was refluxed for 3 h. The solvent was removed under reduced pressure and the resulting residue was purified by silica gel chromatography, eluting with MeOH/EtOAc 1:25; yield 1.1 g, 97%; ^{1}H NMR (MeOH-d_4) δ 3.28 (m, 4H), 4.83 (m, 4H), 8.25 (s, 2H).

4.2.7. 4-Amino-3,5-dinitrobenzenesulfonate (8f). A suspension of **7** (1 g, 3.12 mmol) in a mixture of aq NH_3 (4 mL) and H_2O (8 mL) was refluxed for 2 h, then cooled to rt and kept for crystallization at 0 °C. The crystals were filtered and dried to afford **8f**: 624 mg, 67%; ^{1}H NMR (DMSO-d_6) δ 8.51 (br s, 2H, NH_2), 8.55 (s, 2H, ArH).

4.3. General procedure for the synthesis of N^4,N^4-di-*n*-substituted sulfonates 8g–i

To a suspension of **7** (1 equiv) and *N*,*N*-dialkylamine hydrochloride (1.1 equiv) in methanol, triethylamine (6 equiv) was added and refluxed for 3 h, after which

the solvent was evaporated. The residue was extracted with EtOAc, washed with water and saturated brine, respectively, and dried using anhydrous $MgSO_4$ and evaporated. The product was purified by silica gel column chromatography using a solvent mixture of EtOAc/MeOH.

4.3.1. 3,5-Dinitro-N^4-(cyclopropylmethyl)-N^4-propylbenzenesulfonate (8g). Reaction of **7** (487 mg, 1.52 mmol) and **6a** (250 mg, 1.67 mmol) in the presence of triethylamine (1.26 mL, 9.12 mmol) in MeOH (45 mL) afforded **8g** as a yellow solid: 480 mg, 79%; ^{1}H NMR (DMSO-d_6) δ 0.05–0.11 (m, 2H), 0.39–0.45 (m, 2H), 0.73–0.92 (m, 4H), 1.38–1.48 (m, 2H), 2.83 (d, J = 6.8 Hz, 2H), 2.92–2.97 (m, 2H), 8.14 (s, 2H).

4.3.2. 3,5-Dinitro-N^4,N^4-di(cyclopropylmethyl)benzenesulfonate (8h). Reaction of **7** (472 mg, 1.47 mmol) and **6b** (250 mg, 1.55 mmol) in the presence of triethylamine (1.2 mL, 8.6 mmol) in MeOH (40 mL) afforded **8h** as a yellow solid: 514 mg, 85%; ^{1}H NMR (DMSO-d_6) δ 0.03–0.09 (m, 4H), 0.36–0.43 (m, 4H), 0.77–0.88 (m, 2H), 2.89 (d, J = 7.0 Hz, 4H), 8.16 (s, 2H).

4.3.3. 3,5-Dinitro-N^4-benzyl-N^4-(cyclopropylmethyl)benzenesulfonate (8i). Reaction of **7** (200 mg, 0.62 mmol) and **6c** (135 mg, 0.69 mmol) in the presence of triethylamine (0.5 mL, 3.6 mmol) in MeOH (20 mL) afforded **8i** as a yellow solid: 190 mg, 69%; ^{1}H NMR (DMSO-d_6) δ −0.05 to 0.01 (m, 2H), 0.33–0.40 (m, 2H), 0.78–0.86 (m, 1H), 2.76 (d, J = 6.6 Hz, 2H), 4.23 (s, 2H), 7.22–7.59 (m, 5H), 8.13 (d, J = 1.2 Hz, 2H).

4.4. General procedure for the synthesis of N^1-aryl-3,5-dinitro-N^4,N^4-di-*n*-substituted sufanilamide derivatives (10–26)

To a suspension of sulfonic acids (**8a**–**i**) in dichloromethane, PCl_5 (2.5 equiv) was added and the reaction mixture was stirred for 2 h for **8a**–**e** and **8g**–**i** and overnight for **8f**. The reaction mixture was washed with water and dried using anhydrous Na_2SO_4. The product 3,5-dinitro-N^4,N^4-di-*n*-substituted sulfonylchlorides **9a**–**i** were used without further purification. Pyridine was then added, the temperature was adjusted to 40 °C, 3–5 equiv of aniline derivative was added and the reaction mixture was stirred for 3 h at 40 °C. Pyridine was then evaporated in vacuo, the dark residue was washed with water and extracted with ethyl acetate and the product was purified by silica gel column chromatography. Recrystallization was performed using a solvent mixture of dichloromethane and hexane.

4.4.1. N^1-(4-Fluoro)phenyl-3,5-dinitro-N^4,N^4-di-*n*-propylsulfanilamide (10). Reaction of the sulfonyl chloride **9a** (670 mg, 1.83 mmol, prepared from **8a**) and 4-fluoroaniline (527 μL, 5.49 mmol) in pyridine (30 mL) at 40 °C afforded **10** as yellow crystals, yield 552 mg, 68%; mp = 91 °C; ^{1}H NMR ($CDCl_3$) δ 0.88 (t, 6H, J = 7.4 Hz), 1.52–1.68 (m, 4H), 2.94–3.03 (m, 4H), 6.49 (br s, 1H), 6.99–7.15 (m, 4H), 8.09 (s, 2H); ^{13}C NMR ($CDCl_3$) δ 11.10, 20.67, 54.02, 116.68 (d, J_{CF} = 22.5 Hz), 125.21 (d, J_{CF} = 8.3 Hz), 128.66, 128.81, 130.99 (d, J_{CF} = 3.0 Hz), 141.78, 144.40, 161.24 (d, J_{CF} = 246.0 Hz); HRMS (ESI) calcd for $C_{18}H_{21}FN_4NaO_6S$ $(M+Na)^+$ 463.1064, measured $(M+Na)^+$ 463.1077. Anal. ($C_{18}H_{21}FN_4O_6S$) C, H, N.

4.4.2. N^1-(3-Fluoro)phenyl-3,5-dinitro-N^4,N^4-di-*n*-propylsulfanilamide (11). The reaction of sulfonylchloride **9a** (730 mg, 1.99 mmol, prepared from **8a**) and 3-fluoroaniline (576 μL, 5.98 mmol) in pyridine (32 mL) at 40 °C afforded **11** as yellow crystals: yield 474 mg, 54%; mp = 117 °C; ^{1}H NMR ($CDCl_3$) δ 0.88 (t, 6H, J = 7.2 Hz), 1.53–1.68 (m, 4H), 2.94–2.99 (m, 4H), 6.60 (br s, 1H), 6.83–6.96 (m, 3H), 7.21–7.35 (m, 1H), 8.17 (s, 2H); ^{13}C NMR ($CDCl_3$) δ 11.07, 20.63, 54.00, 108.80 (d, J_{CF} = 25.5 Hz), 113.03 (d, J_{CF} = 21.0 Hz), 116.74, 128.48, 128.85, 131.05 (d, J_{CF} = 9 Hz), 136.97 (d, J_{CF} = 9.8 Hz), 142.01, 144.32, 163.11 (d, J_{CF} = 246.8 Hz); HRMS (ESI) calcd for $C_{18}H_{21}FN_4NaO_6S$ $(M+Na)^+$ 463.1064, measured $(M+Na)^+$ 463.1070 . Anal. ($C_{18}H_{21}FN_4O_6S$) C, H, N.

4.4.3. N^1-(4-Fluoro)phenyl-3,5-dinitro-N^4,N^4-di-*n*-butylsulfanilamide (12). The reaction of sulfonylchloride **9b** (900 mg, 2.3 mmol, prepared from **8b**) and 4-fluoroaniline (770 μL, 8.01 mmol) in pyridine (38 mL) at 40 °C afforded **12** as yellow crystals: yield 450 mg, 42%; mp = 137 °C; ^{1}H NMR ($CDCl_3$) δ 0.89 (t, 6H, J = 7.4 Hz), 1.22–1.35 (m, 4H), 1.52–1.62 (m, 4H), 2.97–3.06 (m, 4H), 6.49 (br s, 1H), 7.02–7.15 (m, 4H), 8.09 (s, 2H); ^{13}C NMR ($CDCl_3$) δ 13.65, 19.86, 29.37, 52.10, 116.68 (d, J_{CF} = 22.5 Hz), 125.31 (d, J_{CF} = 8.3 Hz), 128.58, 128.85, 130.98 (d, J_{CF} = 3.8 Hz), 141.69, 144.47, 161.28 (d, J_{CF} = 246.8 Hz); HRMS (ESI) calcd for $C_{20}H_{25}FN_4NaO_6S$ $(M+Na)^+$ 491.1377, measured $(M+Na)^+$ 491.1393. Anal. ($C_{20}H_{25}FN_4O_6S$) C, H, N.

4.4.4. N^1-(3-Fluoro)phenyl-3,5-dinitro-N^4,N^4-di-*n*-butylsulfanilamide (13). The reaction of sulfonylchloride **9b** (1.22 g, 3.1 mmol, prepared from **8b**) and 3-fluoroaniline (1.04 mL, 10.8 mmol) in pyridine (50 mL) at 40 °C afforded **13** as yellow crystals: yield 676 mg, 48%; mp = 139 °C; ^{1}H NMR ($CDCl_3$) δ 0.89 (t, 6H, J = 7.4 Hz), 1.22–1.34 (m, 4H), 1.52–1.62 (m, 4H), 2.99 (t, 4H, J = 7.5 Hz), 6.71 (br s, 1H), 6.89–6.96 (m, 3H), 7.27–7.35 (m, 1H), 8.16 (s, 2H); ^{13}C NMR ($CDCl_3$) δ 13.65, 19.86, 29.35, 52.12, 108.97 (d, J_{CF} = 24.8 Hz), 113.16 (d, J_{CF} = 21.0 Hz), 116.88 (d, J_{CF} = 3.0 Hz), 128.60, 128.69, 131.07 (d, J_{CF} = 9.8 Hz), 136.92 (d, J_{CF} = 10.5 Hz), 141.91, 144.44, 163.14 (d, J_{CF} = 246 Hz); HRMS (ESI) calcd for $C_{20}H_{25}FN_4NaO_6S$ $(M+Na)^+$ 491.1377, measured $(M+Na)^+$ 491.1383. Anal. ($C_{20}H_{25}FN_4O_6S$) C, H, N.

4.4.5. N^1-(4-Fluoro)phenyl-3,5-dinitro-N^4,N^4-diisobutylsulfanilamide (14). The reaction of sulfonylchloride **9c** (900 mg, 2.29 mmol, prepared from **8c**) and 4-fluoroaniline (770 μL, 8.01 mmol) in pyridine (38 mL) at 40 °C afforded **14** as yellow crystals: yield 672 mg, 63%; mp = 56–57 °C; ^{1}H NMR ($CDCl_3$) δ 0.89 (d, 12H, J = 6.6 Hz), 1.86–1.99 (m, 2H), 2.79 (d, 4H, J = 6.9 Hz), 6.45 (br s, 1H), 7.01–7.13 (m, 4H), 8.05 (s,

2H); ^{13}C NMR ($CDCl_3$) δ 20.17, 26.66, 60.75, 116.54 (d, J_{CF} = 22.5 Hz), 125.26 (d, J_{CF} = 8.3 Hz), 127.66, 129.18, 131.25, (d, J_{CF} = 3.0 Hz), 141.22, 143.43, 161.16 (d, J_{CF} = 245.3 Hz); HRMS (ESI) calcd for $C_{20}H_{25}FN_4NaO_6S$ $(M+Na)^+$ 491.1377, measured $(M+Na)^+$ 491.1360. Anal. ($C_{20}H_{25}FN_4O_6S$) C, H, N.

4.4.6. N^1-(3-Fluoro)phenyl-3,5-dinitro-N^4,N^4-diisobutylsulfanilamide (15). The reaction of sulfonylchloride **9c** (1.03 g, 2.62 mmol, prepared from **8c**) and 3-fluoroaniline (880 μL, 9.17 mmol) in pyridine (43 mL) at 40 °C afforded **15** as yellow crystals: yield 682 mg, 56%; mp = 113–114 °C; ^{1}H NMR ($CDCl_3$) δ 0.89 (d, 12H, J = 6.3 Hz), 1.84–1.97 (m, 2H), 2.78 (d, 4H, J = 6.9 Hz), 6.67 (br s, 1H), 6.88–6.99 (m, 3H), 7.26–7.34 (m, 1H), 8.12 (s, 2H); ^{13}C NMR ($CDCl_3$) δ 20.19, 26.67, 60.79, 109.19 (d, J_{CF} = 25.5 Hz), 113.25 (d, J_{CF} = 20.3 Hz), 117.10 (d, J_{CF} = 3.0 Hz), 127.71, 129.09, 131.03 (d, J_{CF} = 9.0 Hz), 136.94 (d, J_{CF} = 9.8 Hz), 141.34, 143.46, 163.14 (d, J_{CF} = 246.8 Hz); HRMS (ESI) calcd for $C_{20}H_{25}FN_4NaO_6S$ $(M+Na)^+$ 491.1377, measured $(M+Na)^+$ 491.1401. Anal. ($C_{20}H_{25}FN_4O_6S$) C, H, N.

4.4.7. N^1-(4-Fluoro)phenyl-3,5-dinitro-N^4,N^4-dibenzylsulfanilamide (16). The reaction of sulfonylchloride **9d** (prepared from **8d**, 540 mg, 1.121 mmol) and 4-fluoroaniline (537 μL, 5.60 mmol) in pyridine (38 mL) at 40 °C afforded **16** as yellow crystals: yield 287 mg, 48%; mp = 139 °C; ^{1}H NMR ($CDCl_3$) δ 4.15 (s, 4H), 6.41 (s, 1H), 6.92–7.33 (m, 14H), 8.09 (s, 2H); ^{13}C NMR ($CDCl_3$) δ 56.96, 116.75 (d, J_{CF} = 23.3 Hz), 125.71 (d, J_{CF} = 8.3 Hz), 128.43, 128.61, 129.16, 130.35, 130.74 (d, J_{CF} = 3.0 Hz), 134.17, 141.21, 145.04, 161.40 (d, J_{CF} = 246.8 Hz); HRMS (ESI) calcd for $C_{26}H_{21}FN_4NaO_6S$ $(M+Na)^+$ 559.1064, measured $(M+Na)^+$ 559.1090. Anal. ($C_{26}H_{21}FN_4O_6S$) C, H, N.

4.4.8. N^1-(3-Fluoro)phenyl-3,5-dinitro-N^4,N^4-dibenzylsulfanilamide (17). The reaction of sulfonylchloride **9d** (prepared from **8d**, 600 mg, 1.24 mmol) and 3-fluoroaniline (550 μL, 5.7 mmol) in pyridine (40 mL) at 40 °C afforded **17** as yellow crystals: yield 250 mg, 38%; mp = 66–67 °C; ^{1}H NMR ($CDCl_3$) δ 4.10 (s, 4H), 6.74 (br s, 1H), 6.80–7.31 (m, 14H), 8.17 (d, J = 0.9 Hz, 2H); ^{13}C NMR ($CDCl_3$) δ 56.93, 109.39 (d, J_{CF} = 25.5 Hz), 113.48 (d, J_{CF} = 21.0 Hz), 117.28 (d, J_{CF} = 3.0 Hz), 128.46, 128.56, 128.60, 129.18, 130.01, 131.13 (d, J_{CF} = 9.0 Hz), 134.02, 136.68 (d, J_{CF} = 10.5 Hz), 141.47, 144.82, 163.13 (d, J_{CF} = 246.8 Hz); HRMS (ESI) calcd for $C_{26}H_{21}FN_4NaO_6S$ $(M+Na)^+$ 559.1064, measured $(M+Na)^+$ 559.1048. Anal. ($C_{26}H_{21}FN_4O_6S$) H, N, C. Calcd: 58.20; found: 57.70.

4.4.9. N^1-Phenyl-3,5-dinitro-N^4,N^4-dibenzylsulfanilamide (18). The reaction of sulfonylchloride **9d** (prepared from **8d**, 600 mg, 1.24 mmol) and aniline (550 μL, 6 mmol) in pyridine (40 mL) at 40 °C afforded **18** as yellow crystals: yield 250 mg, 40%; mp = 64–65 °C; ^{1}H NMR ($CDCl_3$) δ 4.11 (s, 4H), 6.49 (s, 1H), 7.09–7.38 (m, 15H), 8.10 (s, 2H); ^{13}C NMR ($CDCl_3$) δ 56.87, 122.69, 126.80, 128.39, 128.45, 128.58, 129.17, 129.82, 130.65, 134.20, 135.06, 141.21, 144.98; HRMS (ESI) calcd for $C_{26}H_{22}N_4NaO_6S$ $(M+Na)^+$ 541.1158, measured $(M+Na)^+$ 541.1161. Anal. ($C_{26}H_{22}N_4O_6S$) H, N, C. Calcd: 60.22; found: 60.95.

4.4.10. N^1-(4-Fluoro)phenyl-3,5-dinitro-N^4-pyrrolidin-1-ylsulfanilamide (19). The reaction of sulfonyl chloride **9e** (0.95 g, 2.82 mmol, prepared from **8e)** and 4-fluoroaniline (0.8 g, 7.2 mmol) in pyridine (20 mL) at 40 °C afforded **19** as yellow crystals, yield 842 mg, 73%; mp = 201 °C; ^{1}H NMR (MeOH-d_4) δ 2.05 (m, 4H), 3.28 (m, 4H), 7.08 (m, 2H), 7.29 (m, 2H), 8.24 (s, 2H), 9.1 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 25.64, 54.59, 116.67 (d, J_{CF} = 22.5 Hz), 122.74, 124.96 (d, J_{CF} = 8.3 Hz), 129.15, 134.21 (d, J_{CF} = 3 Hz), 138.87, 141.13, 159.61 (d, J_{CF} = 241.5 Hz); HRMS (ESI) calcd for $C_{16}H_{15}FN_4O_6S$ $(M+Na)^+$ 433.0594, measured $(M+Na)^+$ 433.0584. Anal. ($C_{16}H_{15}FN_4O_6S$) C, H, N.

4.4.11. N^1-(4-Hydroxy)phenyl-3,5-dinitro-N^4,N^4-di-*n*-butylsulfanilamide (20). The reaction of sulfonylchloride **9b** (prepared from **8b**, 600 mg, 1.45 mmol) and 4-hydroxyaniline (632 mg, 5.8 mmol) in pyridine (20 mL) at 40 °C afforded **20** as yellow crystals: yield 443 mg, 66%; mp 180 °C; ^{1}H NMR ($CDCl_3$) δ 0.86 (t, J = 7.4 Hz, 6H), 1.19–1.31 (m, 4H); 1.45–1.55 (m, 4H), 2.95 (t, J = 7.5, 4H), 4.86 (s, 1H), 6.35 (br s, 1H), 6.76 (d, J = 8.9, 2H), 6.97 (d, J = 8.9, 2H), 8.02 (s, 2H); ^{13}C NMR ($CDCl_3$) δ 13.65, 19.86, 29.41, 52.10, 116.43, 126.35, 127.59, 128.54, 129.32, 141.52, 144.55, 154.90; HRMS (ESI) calcd for $C_{20}H_{26}N_4NaO_7S$ $(M+Na)^+$ 489.1420, measured $(M+Na)^+$ 489.1416. Anal. ($C_{20}H_{26}N_4O_7S$) C, H, N.

4.4.12. N^1-(3-Hydroxy)phenyl-3,5-dinitro-N^4,N^4-di-*n*-butylsulfanilamide (21). The reaction of sulfonylchloride **9b** (prepared from **8b**, 600 mg, 1.45 mmol) and 3-hydroxyaniline (632 mg, 5.8 mmol) in pyridine (20 mL) at 40 °C afforded **21** as yellow crystals: yield 432 mg, 64%; mp 152 °C; ^{1}H NMR ($CDCl_3$) δ 0.89 (t, J = 7.5 Hz, 6H), 1.24–1.34 (m, 4H), 1.51–1.61 (m, 4H), 2.99 (t, J = 7.5 Hz, 4H), 4.95 (s, 1H), 6.56 (s, 1H), 6.66–6.70 (m, 3H), 7.19 (m, 1H), 8.15 (s, 2H); ^{13}C NMR ($CDCl_3$) δ 13.65, 19.87, 29.37, 52.11, 108.92, 113.45, 113.78, 128.67, 128.94, 130.79, 136.53, 141.80, 144.47, 156.66; HRMS (ESI) calcd for $C_{20}H_{26}N_4NaO_7S$ $(M+Na)^+$ 489.1420, measured $(M+Na)^+$ 489.1431. Anal. ($C_{20}H_{26}N_4O_7S$) C, H, N.

4.4.13. N^1-(2-Hydroxy)phenyl-3,5-dinitro-N^4,N^4-di-*n*-butylsulfanilamide (22). The reaction of sulfonylchloride **9b** (prepared from **8b**, 600 mg, 1.45 mmol) and 2-hydroxyaniline (632 mg, 5.8 mmol) in pyridine (20 mL) at 40 °C afforded **22** as yellow crystals: yield 481 mg, 71%; mp 128 °C; ^{1}H NMR ($CDCl_3$) δ 0.89 (t, J = 7.4 Hz, 6H), 1.28 (m, 4H), 1.51–1.60 (m, 4H), 2.99 (t, J = 7.5 Hz, 4H), 5.52 (s, 1H), 6.65 (s, 1H), 6.86–6.94 (m, 2H), 7.12–7.19 (m, 2H), 8.12 (s, 2H); ^{13}C NMR ($CDCl_3$) δ 13.66, 19.85, 29.38, 52.09, 116.52, 121.74, 122.34, 125.10, 128.37, 128.58, 128.80, 141.66, 144.39, 148.92; HRMS (ESI) calcd for $C_{20}H_{26}N_4NaO_7S$ $(M+Na)^+$ 489.1420, measured $(M+Na)^+$ 489.1414. Anal. ($C_{20}H_{26}N_4O_7S$) C, H, N.

4.4.14. N^1-Phenyl-3,5-dinitrosulfanilamide (23). The reaction of 4-amino-3,5-dinitrosulfonylchloride **9f** (prepared from **8f**, 50 mg, 0.16 mmol) and aniline (54 μL, 0.58 mmol) in pyridine (2 mL) at 40 °C afforded **23** as yellow crystals: yield 30 mg, 54%; mp 197 °C; ^{1}H NMR (DMSO-d_6) δ 7.07–7.13 (m, 3H), 7.26–7.31 (m, 2H), 8.60 (s, 2H), 8.75 (br s, 2H), 10.40 (br s, 1H); ^{13}C NMR (DMSO-d_6) δ 121.67, 123.81, 125.47, 129.89, 131.46, 135.07, 137.33, 143.01; HRMS (ESI) calcd for $C_{12}H_{10}N_4NaO_6S$ $(M+Na)^+$ 361.0219, measured $(M+Na)^+$ 361.0231. Anal. ($C_{12}H_{10}N_4O_6S$) C, H, N.

4.4.15. N^1-(4-Fluoro)phenyl-3,5-dinitro-N^4-(cyclopropylmethyl)-N^4-propylsulfanilamide (24). The reaction of sulfonylchloride **9g** (prepared from **8g**, 475 mg, 1.19 mmol) and 4-fluoroaniline (460 μL, 4.8 mmol) in pyridine (18 mL) at 40 °C afforded **24** as yellow crystals: yield 268 mg, 50%; mp = 80 °C; ^{1}H NMR ($CDCl_3$) δ 0.14–0.20 (m, 2H), 0.56–0.64 (m, 2H), 0.89 (t, 3H, J = 7.3 Hz), 0.99–1.05 (m, 1H), 1.58–1.67 (m, 2H), 2.92 (d, 2H, J = 7.0 Hz), 3.07–3.13 (m, 2H), 6.65 (s, 1H), 7.02–7.16 (m, 4H), 8.13 (s, 2H); ^{13}C NMR ($CDCl_3$) δ 4.39, 9.63, 11.52, 21.20, 54.60, 57.84, 117.03 (d, J_{CF} = 22.8 Hz), 125.51 (d, J_{CF} = 8.5 Hz), 129.09, 129.47, 131.51 (d, J_{CF} = 3.1 Hz), 142.33, 145.11, 161.58 (d, J_{CF} = 245.6 Hz); HRMS (ESI) calcd for $C_{19}H_{21}FN_4NaO_6S$ $(M+Na)^+$ 475.1064, measured $(M+Na)^+$ 475.1066. Anal. ($C_{19}H_{21}FN_4O_6S$) C, H, N.

4.4.16. N^1-(4-Fluoro)phenyl-3,5-dinitro-N^4,N^4-di-(cyclopropylmethyl)sulfanilamide (25). The reaction of sulfonylchloride **9h** (prepared from **8h**, 500 mg, 1.22 mmol) and 4-fluoroaniline (470 μL, 4.9 mmol) in pyridine (18 mL) at 40 °C afforded **25** as yellow crystals: yield 250 mg, 44%; mp = 114 °C; ^{1}H NMR ($CDCl_3$) δ 0.13–0.19 (m, 4H), 0.53–0.61 (m, 4H), 0.95–1.06 (m, 2H), 3.03 (d, 4H, J = 6.6 Hz), 6.60 (s, 1H), 7.02–7.16 (m, 4H); 8.14 (s, 2H); ^{13}C NMR ($CDCl_3$) δ 4.60, 9.82, 58.14, 117.11 (d, J_{CF} = 22.8 Hz), 125.78 (d, J_{CF} = 8.4 Hz), 128.81, 130.14, 131.33 (d, J_{CF} = 2.9 Hz), 142.25, 145.73, 161.71 (d, J_{CF} = 246.31 Hz); HRMS (ESI) calcd for $C_{20}H_{21}FN_4NaO_6S$ $(M+Na)^+$ 487.1064, measured $(M+Na)^+$ 487.1042. Anal. ($C_{20}H_{21}FN_4O_6S$) C, H, N.

4.4.17. N^1-(4-Fluoro)phenyl-3,5-dinitro-N^4-(cyclopropylmethyl)-N^4-benzylsulfanilamide (26). The reaction of sulfonylchloride **9i** (prepared from **8i**, 161 mg, 0.37 mmol) and 4-fluoroaniline (143 μL, 1.50 mmol) in pyridine (6 mL) at 40 °C afforded **26** as yellow crystals: yield 59 mg, 32%; mp = 160 °C; ^{1}H NMR ($CDCl_3$) δ 0.02–0.08 (m, 2H), 0.53–0.59 (m, 2H), 0.99–1.10 (m, 1H), 2.89 (d, J = 6.6 Hz, 2H), 4.36 (s, 2H), 6.66 (s, 1H), 7.01–7.10 (m, 4H), 7.31–7.33 (m, 5H), 8.11 (s, 2H); ^{13}C NMR ($CDCl_3$) δ 4.08, 9.21, 56.12, 58.64, 116.73 (d, J_{CF} = 22.5 Hz), 125.65 (d, J_{CF} = 9.0 Hz), 128.21, 128.35, 128.65, 128.71, 130.20, 130.78 (d, J_{CF} = 3.0 Hz), 134.93, 141.62, 145.20, 161.4 (d, J_{CF} = 246.7 Hz); HRMS (ESI) calcd for $C_{23}H_{21}FN_4NaO_6S$ $(M+Na)^+$ 523.1064, measured $(M+Na)^+$ 523.1072. Anal. ($C_{23}H_{21}FN_4O_6S$) C, H, N.

4.4.18. 4-Dipropylamino-3,5-dinitrobenzoic acid (28a). A suspension of 4-chloro-3,5-dinitrobenzoic acid **27** (5.0 g, 20.0 mmol) and dipropylamine (30 mL) in 150 mL methanol was refluxed for 3 h until the solution become clear. This solution was evaporated to dryness, the residue was dissolved in EtOAc, washed with 5% HCl, then washed with water. The organic layer was dried over anhydrous Na_2SO_4 and the solvent was evaporated to obtain a yellow solid, which was purified by silica column chromatography to obtain 6.1 g (96%) of **28a**. R_f 0.33 (MeOH/dichloromethane 1:8); mp = 133 °C; ^{1}H NMR ($CDCl_3$) δ 0.90 (t, 6H, J = 7.32 Hz), 1.59–1.74 (m, 4H), 2.80–3.35 (m, 4H), 8.51 (s, 2H); ^{13}C NMR ($CDCl_3$) δ 11.16, 20.74, 54.06, 119.35, 131.35, 142.43, 144.70, 167.56; MS (HR) $C_{13}H_{18}N_3O_6$ $(M+H)^+$ calcd: 312.1195, found: 312.1187.

4.4.19. 4-Dibutylamino-3,5-dinitrobenzoic acid (28b). Experimental conditions for the synthesis of **28b** were identical to those described for compound **28a**. Reaction between **27** (4.0 g, 16 mmol) and 40 mL of dibutylamine (30.7 g, 240 mmol) yielded 4.5 g (82%) of compound **28b** as a yellow solid. R_f 0.50 (MeOH/dichloromethane 1:8); mp 139 °C; ^{1}H NMR ($CDCl_3$) δ 0.85 (t, 6H, J = 7.32 Hz), 1.21–1.36 (m, 4H), 1.48–1.62 (m, 4H), 3.01 (t, 4H, J = 7.5 Hz), 8.45 (s, 2H); ^{13}C NMR (DMSO-d_6) δ 13.58, 19.80, 29.37, 52.00, 119.56, 131.30, 142.36, 144.79, 168.19; MS (HR) $C_{15}H_{22}N_3O_6$ $(M+H)^+$ calcd: 340.1508, found: 340.1510.

4.5. General procedure for the synthesis of amide derivatives (29–39)

A mixture of compound **28a** or **28b** (1 equiv), substituted aniline (1.5 equiv), DCC (1.3 equiv), and DMAP (0.3 equiv) in 5 mL of dichloromethane were reacted at room temperature for 3 h. The reaction was quenched by adding 1 mL water, then the organic layer was extracted with EtOAc, washed with water and dried over anhydrous Na_2SO_4. The solid material obtained after evaporation of the organic layer was purified by silica gel column chromatography to obtain target compounds **29**–**39**. Recrystallization of the target compounds was carried out using a solvent mixture of dichloromethane and hexane.

4.5.1. *N*-Phenyl-4-dipropylamino-3,5-dinitrobenzamide (29). Reaction between **28a** (0.50 g, 1.6 mmol) and aniline (0.26 g, 2.8 mmol) yielded 0.46 g (74%) of **29** as orange crystals; R_f 0.53 (EtOAc/hexane 1:4); mp 178 °C; ^{1}H NMR ($CDCl_3$) δ 0.85 (t, 6H, J = 7.32 Hz), 1.50–1.66 (m, 4H), 2.94 (t, 4H, J = 7.5 Hz), 7.08–7.16 (m, 1H), 7.22–7.33 (m, 2H), 7.48–7.58 (m, 2H), 8.24 (s, 1H), 8.33 (s, 2H); ^{13}C NMR ($CDCl_3$) δ 11.08, 20.78, 54.10, 120.76, 125.30, 125.57, 128.45, 129.10, 137.07, 140.99, 145.10, 161.66; MS (HR) $C_{19}H_{22}N_4O_5Na$ $(M+Na)^+$ calcd: 409.1488, found: 409.1520; Anal. ($C_{19}H_{22}N_4O_5$) C, H, N.

4.5.2. *N*-(4-Fluorophenyl)-4-dipropylamino-3,5-dinitrobenzamide (30). Reaction between **28a** (0.50 g, 1.6 mmol) and 4-fluoroaniline (0.26 g, 2.3 mmol) yielded 0.43 g (66%) of **30** as orange crystals. R_f 0.42 (EtOAc/

hexane 1:4); mp 201 °C; ^{1}H NMR (DMSO-d_6) δ 0.80 (t, 6H, J = 7.32 Hz), 1.46–1.55 (m, 4H), 2.94 (t, 4H, J = 7.5 Hz), 7.10–7.22 (m, 2H), 7.65–7.75 (m, 2H), 8.60 (s, 2H), 10.48 (s, 1H); ^{13}C NMR (acetone-d_6) δ 11.41, 20.69, 53.89, 115.26 (d, J_{CF} = 22.5 Hz), 122.11 (d, J_{CF} = 7.64 Hz), 127.04, 128.24, 134.96 (d, J_{CF} = 2.4 Hz), 140.06, 145.82, 161.07, 161.26 (d, J_{CF} = 241.8 Hz); MS (HR) $C_{19}H_{21}FN_4O_5Na$ $(M+Na)^+$ calcd: 427.1394, found: 427.1418; Anal. ($C_{19}H_{21}FN_4O_5$) C, H, N.

4.5.3. *N*-(3-Fluorophenyl)-4-dipropylamino-3,5-dinitrobenzamide (31). Reaction between **28a** (0.50 g, 1.6 mmol) and 3-fluoroaniline (0.26 g, 2.3 mmol) yielded 0.42 g (65%) of **31** as orange crystals; R_f 0.60 (EtOAc/hexane 1:4); mp 160 °C; ^{1}H NMR ($CDCl_3$) δ 0.99 (t, 6H, J = 7.32 Hz), 1.58–1.70 (m, 4H), 3.01 (t, 4H, J = 7 Hz), 6.89–6.95 (m, 1H), 7.26–7.29 (m, 1H), 7.30–7.40 (m, 1H), 7.57–7.62 (m, 1H), 7.81 (s, 1H), 8.34 (s, 2H); ^{13}C NMR ($CDCl_3$) δ 11.06, 20.69, 54.04, 108.24 (d, J_{CF} = 26.4 Hz), 112.15 (d, J_{CF} = 21.1 Hz), 115.78 (d, J_{CF} = 2.9 Hz), 124.87, 128.41, 130.27 (d, J_{CF} = 9.1 Hz), 138.40 (d, J_{CF} = 11.04 Hz), 141.13, 144.96, 161.50, 164.80 (d,J_{CF} = 246.16 Hz); MS (HR) $C_{19}H_{21}FN_4O_5Na$ $(M+Na)^+$ calcd: 427.1394, found: 427.1385; Anal. ($C_{19}H_{21}FN_4O_5$) C, H, N.

4.5.4. *N*-Phenyl-4-dibutylamino-3,5-dinitrobenzamide (32). Reaction of **28b** (0.60 g, 1.8 mmol) and aniline (0.29 g, 3.0 mmol) yielded 0.60 g (80%) of **32** as orange crystals; R_f 0.50 (EtOAc/hexane 1:4); mp 152 °C; ^{1}H NMR ($CDCl_3$) δ 0.86 (t, 6H, J = 7.32 Hz), 1.19–1.32 (m, 4H), 1.53–1.64 (m, 4H), 2.98 (t, 4H, J = 7.5 Hz), 7.10–7.15 (m, 1H), 7.24–7.32 (m, 2H), 7.52–7.55 (m, 2H), 8.17 (s, 1H), 8.33 (s, 2H); ^{13}C NMR ($CDCl_3$) δ 13.70, 19.91, 29.54, 52.11, 120.75, 125.30, 125.53, 128.42, 129.12, 137.05, 140.97, 145.17, 161.66; MS (HR) $C_{21}H_{26}N_4O_5Na$ $(M+Na)^+$ calcd: 437.1801, found: 437.1830; Anal. ($C_{21}H_{26}N_4O_5$) C, H, N.

4.5.5. *N*-(4-Fluorophenyl)-4-dibutylamino-3,5-dinitrobenzamide (33). Reaction between **28b** (0.60 g, 1.8 mmol) and 4-fluoroaniline (0.30 g, 2.7 mmol) yielded 0.52 g (67%) of **33** as red crystals; R_f 0.30 (EtOAc/hexane 1:4); mp 164 °C; ^{1}H NMR ($CDCl_3$) δ 0.85 (t, 6H, J = 7.32 Hz), 1.26–1.36 (m, 4H), 1.51–1.64 (m, 4H), 3.03 (t, 4H, J = 7.5 Hz), 7.00–7.10 (m, 2H), 7.50–7.60 (m, 2H), 7.97 (s, 1H), 8.26 (s, 2H); ^{13}C NMR ($CDCl_3$) δ 13.60, 19.83, 29.45, 52.06, 116.02 (d, J_{CF} = 22.5 Hz), 122.58 (d, J_{CF} = 7.67 Hz), 125.09, 128.24, 132.91 (d, J_{CF} = 2.9 Hz), 140.98, 145.12, 161.45, 161.81(d, J_{CF} = 244.72 Hz); MS (HR) $C_{21}H_{25}FN_4O_5Na$ $(M+Na)^+$ calcd: 455.1707, found: 455.1693; Anal. ($C_{21}H_{25}FN_4O_5$) C, H, N.

4.5.6. *N*-(3-Fluorophenyl)-4-dibutylamino-3,5-dinitrobenzamide (34). Reaction between **28b** (0.60 g, 1.8 mmol) and 3-fluoroaniline (0.30 g, 2.7 mmol) yielded 0.53 g (68%) of compound **34** as orange crystals; R_f 0.33 (EtOAc/hexane 1:4); mp 154 °C; ^{1}H NMR ($CDCl_3$) δ 0.83 (t, 6H, J = 7.32 Hz), 1.20–1.32 (m, 4H), 1.52–1.64 (m, 4H), 3.24 (t, 4H, J = 7.5 Hz), 6.80–6.90 (m, 1H), 7.20–7.33 (m, 2H), 7.49–7.57 (m, 1H), 7.97 (s, 1H), 8.32 (s, 2H); ^{13}C NMR ($CDCl_3$) δ 13.60, 19.82, 29.43, 52.07, 108.19 (d, J_{CF} = 25.9 Hz), 112.11 (d, J_{CF} = 21.6 Hz), 115.71 (d, J_{CF} = 2.9 Hz), 124.87, 128.32, 130.29 (d, J_{CF} = 9.1 Hz), 138.57 (d, J_{CF} = 11.04 Hz), 141.09, 145.05, 161.41, 164.83 (d, J_{CF} = 245.7 Hz); MS (HR) $C_{21}H_{25}FN_4O_5Na$ $(M+Na)^+$ calcd: 455.1707, found: 455.1692; Anal. ($C_{21}H_{25}FN_4O_5$) C, H, N.

4.5.7. *N*-(3-Chlorophenyl)-4-dibutylamino-3,5-dinitrobenzamide (35). Reaction between **28b** (0.50 g, 1.5 mmol) and 3-chloroaniline (0.37 g, 3.0 mmol) yielded 0.36 g (53%) of **35** as yellow crystals; R_f0.47 (EtOAc/hexane 1:4); mp 145 °C; ^{1}H NMR ($CDCl_3$) δ 0.86 (t, 6H, J = 7.32 Hz), 1.19–1.32 (m, 4H), 1.49–1.61 (m, 4H), 3.00 (t, 4H, J = 7.5 Hz), 6.95–6.98 (m, 1H), 7.16–7.22 (m, 1H), 7.32–7.42 (m, 2H), 7.87 (s, 1H), 8.32 (s, 2H); ^{13}C NMR ($CDCl_3$) δ 13.71, 19.92, 29.52, 52.15, 118.54, 120.69, 124.83, 125.36, 128.48, 130.17,134.87, 138.20, 141.22, 145.08, 161.52; MS (HR) $C_{21}H_{25}ClN_4O_5Na$ $(M+Na)^+$ calcd: 470.1411, found: 470.1408; Anal. ($C_{21}H_{25}ClN_4O_5$) C, H, N.

4.5.8. *N*-(3-Methoxyphenyl)-4-dibutylamino-3,5-dinitrobenzamide (36). Reaction between **28b** (0.50 g, 1.5 mmol) and *m*-anisidine (0.36 g, 3.0 mmol) yielded 0.37 g (55%) of **36** as orange crystals; R_f 0.45 (EtOAc/hexane 1:4); mp 125 °C; ^{1}H NMR ($CDCl_3$) δ 0.85 (t, 6H, J = 7.32 Hz), 1.19–1.32 (m, 4H), 1.49–1.62 (m, 4H), 3.02 (t, 4H, J = 7.5 Hz), 3.79 (s, 3H), 6.70 (dd, 1H, J = 7.80 Hz, J = 2.47 Hz), 7.04–7.10 (m, 1H), 7.20–7.27 (m, 1H), 7.28–7.31 (m, 1H), 7.90 (s, 1H), 8.32 (s, 2H); ^{13}C NMR ($CDCl_3$) δ 13.72, 19.93, 29.55, 52.14, 55.34, 106.44, 110.98, 112.69, 125.50, 128.36, 129.87, 138.25, 141.02, 145.18, 160.23, 161.49; MS (HR) $C_{22}H_{28}N_4O_6Na$ $(M+Na)^+$ calcd: 467.1907, found: 467.1905; Anal. ($C_{22}H_{28}N_4O_6$) C, H, N.

4.5.9. *N*-(*m*-Tolyl)-4-dibutylamino-3,5-dinitrobenzamide (37). Reaction between **28b** (0.60 g, 1.8 mmol) and *m*-toluidine (0.37 g, 3.5 mmol) yielded 0.40 g (52%) of **37** as yellow crystals; R_f 0.47 (EtOAc/hexane 1:4); mp 125 °C; ^{1}H NMR ($CDCl_3$) δ 0.88 (t, 6H, J = 7.32 Hz), 1.20–1.35 (m, 4H), 1.48–1.62 (m, 4H), 2.25 (s, 3H), 3.01 (t, 4H, J = 7.5 Hz), 6.93–6.99 (m, 1H), 7.16–7.24 (m, 1H), 7.32–7.35 (m, 1H), 7.43 (s, 1H), 8.30 (s, 1H), 8.32 (s, 2H); ^{13}C NMR ($CDCl_3$) δ 13.72, 19.93, 21.44, 29.55, 52.12, 117.72, 121.30, 125.66, 126.10, 128.34, 128.96, 136.99, 139.17, 140.95, 145.20, 161.49; MS (HR) $C_{22}H_{28}N_4O_5Na$ $(M+Na)^+$ calcd: 451.1957, found: 451.1953; Anal. ($C_{22}H_{28}N_4O_5$) C, H, N.

4.5.10. *N*-(3,4-Dichlorophenyl)-4-dibutylamino-3,5-dinitrobenzamide (38). Reaction between **28b** (0.60 g, 1.8 mmol) and 3,4-dichloroaniline (0.47 g, 3.0 mmol) yielded 0.56 g (64%) of **37** as yellow crystals; R_f0.55 (EtOAc/hexane 1:4); mp 150 °C; ^{1}H NMR ($CDCl_3$) δ 0.86 (t, 6H, J= 7.32 Hz), 1.19–1.32 (m, 4H), 1.54–1.65 (m, 4H), 2.99 (t, 4H, J = 7.5 Hz), 7.35–7.40 (m, 1H), 7.42–7.47 (m, 1H), 7.78–7.80 (m, 1H), 8.23 (s, 1H), 8.34 (s, 2H); ^{13}C NMR (acetone-d_6) δ 13.07, 19.62, 29.62, 51.97, 119.99, 121.68, 126.49, 126.52, 128.47, 130.54, 131.77, 138.74, 140.31, 145.92, 161.68; MS (HR) $C_{21}H_{24}Cl_2N_4O_5Na$ $(M+Na)^+$ calcd: 505.1021, found: 505.1006; Anal. ($C_{21}H_{24}Cl_2N_4O_5$) C, H, N.

4.5.11. *N*-(3,5-Dichlorophenyl)-4-dibutylamino-3,5-dinitrobenzamide (39). Reaction between **28b** (0.50 g, 1.5 mmol) and 3,5-dichloroaniline (0.48 g, 3.0 mmol) yielded 0.41 g (57%) of **37** as red crystals; R_f 0.45 (EtOAc/hexane 1:4); mp 170 °C; ^{1}H NMR ($CDCl_3$) δ 0.91 (t, 6H, J = 7.32 Hz), 1.23–1.38 (m, 4H), 1.48–1.63 (m, 4H), 3.05 (t, 4H, J = 7.5 Hz), 7.20–7.22 (m, 1H), 7.62 (s, 1H), 7.63 (s, 1H), 7.80 (s, 1H), 8.35 (s, 2H); ^{13}C NMR ($CDCl_3$) δ 13.71, 19.92, 29.51, 52.18, 118.67, 124.28, 125.25, 128.46, 135.53, 138.88, 141.40, 145.06, 161.39; MS (HR) $C_{21}H_{24}Cl_2N_4O_5Na$ $(M+Na)^+$ calcd: 505.1021, found: 505.1028; Anal. ($C_{21}H_{24}Cl_2N_4O_5$) C, H, N.

4.6. Cell based assays

Assays used to measure the activity of the target compounds against *L. donovani* axenic amastigotes,[13,22] *T. b. rhodesiense* bloodstream forms[23,24] and intracellular *T. cruzi*[23,24] have been described previously. Methods for measuring the toxicity of the compounds against Vero cells[25] and L6 rat myoblasts[23,24] have also been outlined earlier.

4.7. Tubulin assays

Tubulin from *Leishmania tarentolae* was purified according to the general method of Werbovetz et al.[22] with some modifications (A. Yakovich et al., submitted). The purification of mammalian tubulin and methods to assay the effects of target compounds against the assembly of leishmanial and mammalian tubulin have been described previously.[14]

4.8. In vitro metabolism

Triplicate rat liver S9 (BD Biosciences, San Jose, CA) reaction mixtures (0.1 M phosphate, 1 mg/mL S9, 1 mM NADPH, pH 7.4) were incubated at 37 °C for 5 min before adding the test compound at a final concentration of 300 nM (final reaction volume = 330 μL). Triplicate control reactions were produced by replacing S9 with bovine serum albumin (BSA). Reactions were mixed by pipetting up and down for 10 s. Aliquots (100 μL) were withdrawn immediately at 10 min and added directly to 1 mL acetonitrile at 4 °C containing 10 nM N^1-(3,5-dichloro)phenyl-3,5-dinitro-N^4,N^4-di-*n*-propylsulfanilamide[14] internal standard (IS). Samples were then centrifuged at 16,000*g* for 10 min and the supernatant was recovered and dried in a vacuum concentrator with vapor trap (Thermo Electron, Waltham, MA). Samples were resuspended in 150 μL of 50% acetonitrile, centrifuged as above, and the supernatant was loaded for analysis. An LC/MS/MS method was created for each compound using an Agilent 1100 LC system and a ThermoFinnigan TSQ Quantum Discovery Max Mass Spectrometer. Samples were separated on a reversed-phase gradient of water and acetonitrile, each containing 0.1% acetic acid using an Agilent Zorbax extended C18 column (2.1 × 50 mm, 3.5 μm) and analyzed via electrospray ionization in negative ion mode with single reaction monitoring of the parent compound. Standard curves were generated in control reaction mixtures (with BSA) for each LC/MS/MS run. The linear range for each compound covered 2–3 orders of magnitude of concentration with lower limits of quantitation between 3 and 10 nM. Triplicate experiments were completed twice for a total of six replicates per compound.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2006.04.017

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