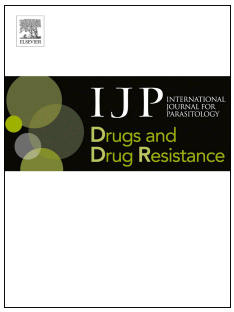

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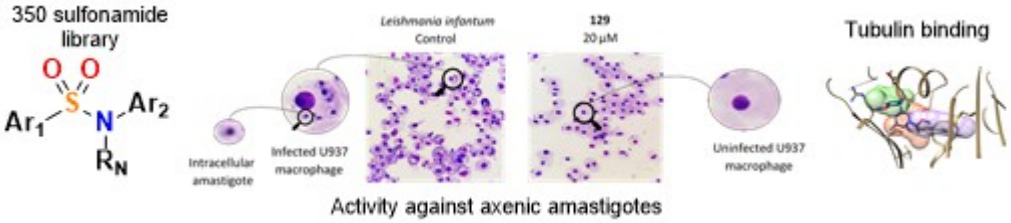

New diarylsulfonamide inhibitors of *Leishmania infantum* amastigotes

Myriam González[a,b,c], Pedro José Alcolea[d], Raquel Álvarez[a,b,c], Manuel Medarde[a,b,c], Vicente Larraga[d], Rafael Peláez[a,b,c*]

[a] Laboratorio de Química Orgánica y Farmacéutica, Departamento de Ciencias Farmacéuticas, Facultad de Farmacia, Universidad de Salamanca, Salamanca, Spain.

[b] Instituto de Investigación Biomédica de Salamanca (IBSAL), Facultad de Farmacia, Universidad de Salamanca, Salamanca, Spain.

[c] Centro de Investigación de Enfermedades Tropicales de la Universidad de Salamanca (CIETUS), Facultad de Farmacia, Universidad de Salamanca, Salamanca, Spain.

[d] Laboratorio de Parasitología Molecular, Departamento de Microbiología Molecular y Biología de las Infecciones, Centro de Investigaciones Biológicas Margarita Salas, Consejo Superior de Investigaciones Científicas, Madrid, Spain.

[*]pelaez@usal.es (RP)

Abstract

New drugs against visceral leishmaniasis with mechanisms of action differing from existing treatments and with adequate cost, stability, and properties are urgently needed. No antitubulin drugs are currently in the clinic against *Leishmania infantum*, the causative agent of visceral leishmaniasis in the Mediterranean area. We have designed and synthesized a focused library of 350 compounds against the *Leishmania* tubulin based on the known structure-activity relationship (SAR) and the sequence differences between the hosts and the parasite and we have ascertained that the synthesized compounds are accessible, stable, and with acceptable water solubility. We have assayed the library against *Leishmania* promastigotes, axenic, and intracellular amastigotes and found 0, 8, and 16 active compounds, respectively, with a high success rate against intracellular amastigotes of over 10 % after discarding the cytotoxic compounds. Five compounds were of similar or better potency than the clinically used miltefosine. 14 compounds showed a host-dependent mechanism of action that might be advantageous as it may render them less susceptible to the development of drug resistance. The active compounds cluster in five chemical classes that provide structure-activity relationships for further hit improvement and facilitate the development of the series. Molecular docking is consistent with the proposed mechanism of action, is supported by the observed structure-activity relationships, and suggests a potential extension to other *Leishmania* species due to the observed sequence similarities. A new family of diarylsulfonamides designed against the parasite tubulins was shown to be active against *Leishmania infantum* and represents a new class of potential drugs with favorable cost, stability, and aqueous solubility for the treatment of visceral leishmaniasis (VL). These results could be extended to other clinically relevant species of *Leishmania* spp.

Keywords

Leishmania; amastigote; sulfonamides; tubulin.

1. Introduction

Leishmaniasis is a neglected tropical disease caused by protozoan parasites classified into the genus *Leishmania* (Kinetoplastida: Trypanosomatidae). The incidence is 700,000-2,000,000 cases causing 20,000-30,000 annual deaths (Alvar et al., 2012). The main clinical forms are kala-azar or visceral (VL), cutaneous (CL), and mucocutaneous leishmaniasis (MCL). VL is fatal without treatment. Anthroponotic VL (AVL) is caused by *Leishmania donovani* in Southeastern Asia and Western Africa, whereas zoonotic VL (ZVL) is caused by *Leishmania infantum* and distributes in the Mediterranean basin and South America. Dogs are the main reservoirs of ZVL, whose incidence is limited in humans in developed countries. Nevertheless, about a decade ago, an important outbreak in humans was registered in Spain (Arce et al., 2013; Jiménez et al., 2014; Molina et al., 2012).

The life cycle of *Leishmania* spp. is digenetic and develops in two stages. The promastigote is a motile fusiform extracellular stage and the amastigote is a round intracellular stage whose flagellum does not emerge from the cellular body. Promastigotes undergo a differentiation process known as metacyclogenesis within the sand fly vector (Diptera: Psychodidae) gut. The vector injects highly infective forms called metacyclic promastigotes in the mammalian host's dermis during blood feeding. Metacyclic promastigotes are internalized by phagocytes and differentiate into amastigotes, which multiply within infected cells, affecting different tissues depending on the causative species. When a sand fly feeds on an infected host amastigotes transform into procyclic promastigotes within the peritrophic membrane and begin differentiation into metacyclic forms as they migrate towards the anterior midgut (Alcolea et al., 2016; Escudero-Martínez et al., 2017).

The number of drugs available against the parasite is limited and they present toxicity, side effects, resistance (Jain and Jain, 2018), long-term treatment, and cost limitations [reviewed in (Nagle et al., 2014; Ponte-Sucre et al., 2017; Rama et al., 2015)]. Their efficacy is variable, depending on the species, the clinical development these species cause, and the host (Tiuman et al., 2011). Combination therapy of current

treatments is being explored leading to moderate improvement (Zulfiqar et al., 2017). Hence, new drugs are required to control this challenging disease (Zhang et al., 2018). The most used drugs during the last 70 years have been pentavalent antimonials, administered by the intramuscular or the intravenous route. The long-term treatments cause serious side effects, including cardiac arrhythmia and acute pancreatitis (Monzote, 2009; Nagle et al., 2014). Efficacy has decreased over time by resistance, associated with multidrug resistance phenotypes (Légaré et al., 2001), mutations in the macrophage aquaporin AQP1 gene, and IL10-mediated up-regulation of the macrophage multiple resistance protein MDR1 (Marquis et al., 2005). Amphotericin B is a polienic macrolide antibiotic with powerful antifungal and antileishmanial activity. This drug also causes important side effects, is expensive, poorly soluble in water, not stable in the gastric environment, and poorly membrane permeable. Fungizone®, a micellar suspension of sodium deoxycholate is administered by the intravenous route and the patients must be hospitalized and monitored (Abu Ammar et al., 2019; Monzote, 2009; Nagle et al., 2014). In the 1990s it replaced pentamidine as second-line therapy for refractory VL cases in India (Nagle et al., 2014). High-cost lipid formulations (AmBisome®) allow lower dosages and side effects, and are successfully used in the control of VL in the Indian subcontinent, but ineffective against other species in other countries (De Rycker et al., 2018). Resistance has emerged associated with changes in ergosterol biosynthesis and oxidative stress prevention (Mbongo et al., 1998). Miltefosine was approved as a first-line drug in 2002 to replace antimonials in several regions (Nagle et al., 2014; Sundar et al., 2002). It initially showed powerful antileishmanial activity, but a gradual increase in resistances related to transporters (Mondelaers et al., 2016; Pérez-Victoria et al., 2006, 2003) and relapses has followed (Rijal et al., 2013; Sundar and Murray, 2005). Paromomycin as an ointment works against CL but is not frequently used due to its side effects (e.g. ototoxicity) (Monzote, 2009; Sundar et al., 2007). Several drug classes, such as the aminopyrazoles, the nitroimidazoles, the oxaboroles, the proteasome inhibitors, and the kinase inhibitors, are currently in development against VL, but oral, safe, effective, low cost, and of short course administration new chemotypes acting on alternative targets are still required (Alves et al., 2018).

The sulfonamides are synthetically accessible, stable, drug-like compounds that have a long history of clinical success (Drews, 2000). Their antiparasitic and antitumor effects have been linked to inhibition of

the microtubule dynamics (Dumontet and Jordan, 2010; Vicente-Blázquez et al., 2019). None of the current antileishmanial drugs in the clinical practice or clinical trials target tubulin. Diarylsulfonamides bind at the colchicine site of tubulin, inhibiting microtubule dynamics, and eliciting antimitotic activity (Vicente-Blázquez et al., 2019). They combine being a privileged scaffold for the generation of pharmacological activities with synthetic accessibility and adequate pharmacokinetic profiles, arising from a favorable combination of chemical stability, hydrogen bonding ability, polarity, hydrophilic-lipophilic balance, adjustable pK_a values, solubility, and conformational preferences (Alcolea et al., 2010; Laurence et al., 2009; Perlovich et al., 2014).

The microtubules of eukaryotic cells are made up of αβ-tubulin dimers, and most drugs affecting microtubule dynamics bind to the tubulin dimer, the microtubule lattice, or microtubule-associated proteins and motors. Tubulins are highly conserved throughout evolution, but certain differences between the mammal and parasite orthologs suggest sufficient binding selectivity for drug development. These cytoskeletal supramolecular structures are involved in structural support, cell motility, cell division, organelle transport, maintenance of cell morphology, and signal transduction (Jordan et al., 1998). Specifically, *Leishmania* tubulin is an essential component of the flagellum and the subpellicular microtubules. These structures are related to parasite survival (Sinclair and de Graffenried, 2019; Sunter and Gull, 2017). At least seven distinct drug-binding sites have been identified in tubulin and the microtubules, named after their prototypical drugs: the taxanes, the Vinca minor alkaloids, the maitansin, the peloruside/laulimalide, the eribulin, the pironetin, and the colchicine binding sites (Vicente-Blázquez et al., 2019). Different parasitic species show sequence variations compared to their hosts which vary depending on the sites, thus making them more or less susceptible to specific drug classes and representatives, thus allowing for specific treatments (Dostál and Libusová, 2014). *Leishmania* parasites are not susceptible to colchicine (Luis et al., 2013), the archetypical ligand of the mammalian eponymous domain. Hence, an opportunity for selective ligand development arises. This has also been empirically exploited in the treatment of helminth and fungal parasitosis with antimitotic benzimidazoles binding at the colchicine site, such as triclabendazole or albendazole (Lacey, 1990).

2. Materials and methods

2.1 Chemical synthesis

General chemical techniques. Reagents were used as purchased without further purification. Solvents (THF, DMF, CH_2Cl_2, toluene) were dried and freshly distilled before use according to procedures described in the literature. TLC was performed on precoated silica gel polyester plates (0.25 mm thickness) with a UV fluorescence indicator 254 (Polychrom SI F254). Chromatographic separations were performed on silica gel columns by flash (Kieselgel 40, 0.040-0.063; Merck) chromatography. Melting points were determined on a Buchi 510 apparatus and are uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded in $CDCl_3$, CD_3OD, or Acetone-d_6 on a Bruker WP 200-SY spectrometer at 200/50 MHz or a Bruker SY spectrometer at 400/100 MHz. Chemical shifts (δ) are given in ppm downfield from tetramethylsilane and coupling constants (J values) are in Hertz. IR spectra were run on a Nicolet Impact 410 Spectrophotometer. Electrospray-ionisation (ESI) high-resolution mass spectra (HRMS) were obtained on a VG-TS250 apparatus (70 eV). A Helios-α UV-320 from Thermo-Spectronic was used for UV spectra.

1,4-dimethoxy-2-nitrobenzene (74). To a solution of 1,4-dimethoxybenzene (2.35 g, 17 mmol) in acetic acid (30 mL) at 0°C, nitric acid (1.13 mL, 17 mmol) in acetic acid (20 mL) was added dropwise under nitrogen atmosphere. The reaction mixture was stirred at 0°C for 4 h and then poured onto ice with $NaHCO_3$ 5% and extracted with ethyl acetate. The organic layers were washed to neutrality with brine, dried over anhydrous Na_2SO_4, filtered and concentrated in vacuum to obtain 2.92 g (94%) of **74**. M.p.: 71.8-72.5 °C (CH_2Cl_2/Hexane). IR (KBr): 1528, 874, 763 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.82 (3H, *s*), 3.92 (3H, *s*), 7.03 (1H, *d*, *J* = 9.6), 7.12 (1H, *dd*, *J* = 9.6 and 3.2), 7.4 (1H, *d*, *J* = 3.2). ^{13}C NMR (100 MHz, $CDCl_3$): δ 55.9 (CH_3), 56.9 (CH_3), 109.9 (CH), 115.0 (CH), 120.8 (CH), 139.3 (C), 147.3 (C), 152.7 (C). GC-MS ($C_8H_9NO_4$): calcd 183, found 183.

2,5-dimethoxyaniline (75). 1,4-dimethoxy-2-nitrobenzene (**74**, 2.92 g, 15.95 mmol) was suspended in ethyl acetate (100 mL) and was palladium-catalyzed (Pd (C) 10 mg) reduced under H_2 atmosphere for 48 h. The reaction mixture was filtered through celite and the solvent evaporated in vacuum to isolate 2.42 g

(99%) of **75**. Crude reaction product was obtained and used without further purification. IR (KBr): 3459, 1519, 839 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.72 (3H, *s*), 3.79 (3H, *s*), 6.24 (1H, *dd*, *J* = 9.2 and 3.2), 6.33 (1H, *d*, *J* = 3.2), 6.69 (1H, *d, J* = 9.2). ^{13}C NMR (100 MHz, $CDCl_3$): δ 54.5 (CH_3), 55.1 (CH_3), 100.9 (CH), 101.1 (CH), 110.3 (CH), 136.2 (C), 140.9 (C), 153.3 (C). GC-MS ($C_8H_{11}NO_2$): calcd 153, found 153.

***N*-(2,5-dimethoxyphenyl)-4-methoxybenzenesulfonamide (76).** To a solution of **75** (2.42 g, 15.84 mmol) in CH_2Cl_2 (50 mL) and pyridine (2 mL), was slowly added 4-methoxybenzenesulfonyl chloride (3.27 g, 15.84 mmol). The mixture was stirred at room temperature for 4 h. Then the reaction was treated with HCl 2N and $NaHCO_3$ 5%, washed with brine, dried over anhydrous Na_2SO_4 and the solvent evaporated to obtain 4.9 g (95%) of **76**. It was purified by crystallization in CH_2Cl_2/Hexane (4.29 g, 84%). M.p.: 114-115 °C (CH_2Cl_2/Hexane). IR (KBr): 3313, 1578, 830 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.62 (3H, *s*), 3.74 (3H, *s*), 3.81 (3H, *s*), 6.53 (1H, *dd, J* = 9.2 and 3.2), 6.65 (1H, *d, J* = 9.2), 6.86 (2H, *d, J* = 9.2), 7.01 (1H, *s*), 7.14 (1H, *d, J* = 3.2), 7.72 (2H, *d, J* = 9.2). ^{13}C NMR (100 MHz, $CDCl_3$): δ 55.5 (CH_3), 55.7 (CH_3), 56.2 (CH_3), 196.8 (CH), 109.5 (CH), 111.4 (CH), 113.9 (2CH), 126.8 (C), 129.4 (2CH), 130.7 (C), 143.4 (C), 153.8 (C), 163.0 (C). HRMS ($C_{15}H_{17}NO_5S + H^+$): calcd 324.0900 ($M + H^+$), found 324.0900.

***N*-(2,5-dimethoxy-4-nitrophenyl)-4-methoxybenzenesulfonamide (96).** To a stirred solution at 0°C of **76** (2.07 g, 6.42 mmol) in acetic acid (30 mL), nitric acid (0.44 mL, 6.42 mmol) in acetic acid (20 mL) was slowly added under nitrogen atmosphere. After 4 h at 0°C, the reaction mixture was poured onto ice and basified with 5% $NaHCO_3$ solution. Then it was extracted with ethyl acetate. The organic layers were washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated in vacuum to obtain 2.14 g (90%) of **96**. By crystallization in CH_2Cl_2/Hexane 1.53 g (65%) of purified product were isolated. M.p.: 161-163 °C (CH_2Cl_2/Hexane). IR (KBr): 3277, 1522, 1450, 822 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.76 (3H, *s*), 3.79 (3H, *s*), 3.88 (3H, *s*), 6.9 (2H, *d, J* = 9.2), 7.26 (1H, *s*), 7.39 (1H, *s*), 7.56 (1H, *s*), 7.77 (2H, *d, J* = 9.2). ^{13}C NMR (100 MHz, $CDCl_3$): δ 55.7 (CH_3), 56.6 (CH_3), 57.0 (CH_3), 103.2 (CH), 108.3 (CH), 114.4 (2CH), 129.4 (2CH), 129.9 (C), 132.8 (C), 133.1 (C), 141.1 (C), 149.2 (C), 163.6(C). HRMS ($C_{15}H_{16}N_2O_7S + H^+$): calcd 369.0751 ($M + H^+$), found 369.0753.

***N*-(4-amino-2,5-dimethoxyphenyl)-4-methoxybenzenesulfonamide (104).** The nitro sulfonamide **96** (1.44 g, 3.91 mmol) in ethyl acetate (100 mL) and Pd (C) (10 mg) was stirred at room temperature under H_2 atmosphere for 48 h. By filtration through celite and solvent evaporation, hydrogenated sulfonamide **104** (1.28 g, 97%) was obtained. 1,11 g (84%) of **104** were isolated by crystallization in CH_2Cl_2/Hexane. M.p.: 164-166 °C (CH_2Cl_2/Hexane). IR (KBr): 3430, 3291, 1451, 834 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 3.38 (3H, *s*), 3.79 (3H, *s*), 3.82 (3H, *s*), 6.15 (1H, *s*), 6.54 (1H, *s*), 6.82 (2H, *d, J* = 9.2), 7.04 (1H, *s*), 7.58 (2H, *d, J* = 9.2). ^{13}C NMR (100 MHz, $CDCl_3$): δ 55.5 (CH_3), 55.9 (CH_3), 56.2 (CH_3), 99.2 (CH), 108.4 (CH), 113.5 (2CH), 115.3 (C), 129.4 (2CH), 130.7 (C), 134.6 (C), 141.0 (C), 145.8 (C), 162.7 (C). HRMS ($C_{15}H_{18}N_2O_5S + H^+$): calcd 339.1009 ($M + H^+$), found 339.1012.

2-chloro-*N*-(2,5-dimethoxy-4-((4-methoxyphenyl)sulfonamido)phenyl)acetamide (129). To a solution of amine **104** (160 mg, 0.47 mmol) in CH_2Cl_2 (25 mL) 2-chloroacetyl chloride (46.4 µL, 0.57 mmol) was added dropwise under nitrogen atmosphere. After 12 h at room temperature the reaction was washed with water, dried over anhydrous Na_2SO_4 and the solvent evaporated in vacuum to give 171 mg (87%) of **129.** The crude reaction product was purified by crystallization in methanol (55 mg, 28%). M.p.: 175-177 °C (MeOH). IR (KBr): 3372, 3265, 1677, 1598, 829 cm^{-1}. ^1H NMR (400 MHz, CD_3OD): δ 3.47 (3H, *s*), 3.81 (3H, *s*), 3.86 (3H, *s*), 4.25 (2H, *s*), 6.94 (2H, *d, J* = 8.8), 7.14 (1H, *s*), 7.62 (2H, *d, J* = 8.8), 7.74 (1H, *s*). ^{13}C NMR (100 MHz, Acetone-d_6): δ 43.2 (CH_2), 55.1 (CH_3), 55.8 (CH_3), 56.0 (CH_3), 104.1 (CH), 106.9 (CH), 113.8 (2CH), 124.8 (C), 129.3 (2CH), 131.4 (C), 138.1 (C), 142.4 (C), 144.9 (C), 163.0 (C), 164.1 (C). HRMS ($C_{17}H_{19}ClN_2O_6S + H^+$): calcd 415.0725 ($M + H^+$), found 415.0700.

3-chloro-*N*-(2,5-dimethoxy-4-((4-methoxyphenyl)sulfonamido)phenyl)propanamide (138). To a stirred solution at room temperature of **104** (143 mg, 0.42 mmol) in CH_2Cl_2 (30 mL) 3-chloropropanoyl chloride (49.4 µL, 0.51 mmol) was slowly added under nitrogen atmosphere. After 12 h, the reaction mixture was crystalized in CH_2Cl_2 to obtain 88 mg (48%) of **138**. M.p.: 193-197 °C (CH_2Cl_2). ^1H NMR (400 MHz, CD_3OD): δ 2.89 (2H, *t, J* = 6.4), 3.47 (3H, *s*), 3.81 (3H, *s*), 3.83 (3H, *s*), 3.83 (2H, *t, J* = 6.4), 6.95 (2H, *d, J* = 9.2), 7.12 (1H, *s*), 7.63 (2H, *d, J* = 9.2), 7.68 (1H, *s*). ^{13}C NMR (100 MHz, Acetone-d_6): δ 39.6 (CH_2), 40.1 (CH_2), 55.1 (CH_3), 55.7 (CH_3), 55.9 (CH_3), 104.4 (CH), 107.1 (CH), 113.7 (2CH), 120.9 (C), 125.7 (C), 129.3

(2CH), 131.7 (C), 142.2 (C), 144.9 (C), 162.9 (C), 167.8 (C). HRMS ($C_{18}H_{21}ClN_2O_6S + H^+$): calcd 429.0882 (M + H^+), found 429.0879.

4-methoxy-3-nitrobenzonitrile (250). To a solution of 4-methoxy-3-nitrobenzaldehyde (870 mg, 4.80 mmol) in MeOH (30 mL) hydroxylamine hydrochloride (334 mg, 4.80 mmol) and two drops of pyridine were added. After 24 h at reflux, the solvent was evaporated, the obtained residue was dissolved in CH_2Cl_2 and washed with water, dried over anhydrous Na_2SO_4, evaporated under vacuum, and dissolved in 15 mL of pyridine. Finally, acetic anhydride (1 mL) was added to the mixture. After 9 h at room temperature, the reaction was treated with HCl 2N, extracted with CH_2Cl_2 and the solvent evaporated to obtain 685 mg (80%) of **250** Crude reaction product was obtained and used without further purification. ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.05 (3H, *s*), 7.20 (1H, *d, J* = 8.8), 7.83 (1H, *dd, J* = 8.8 and 2), 8.15 (1H, *d, J* = 2). GC-MS ($C_8H_6N_2O_3$): calcd 178, found 178.

3-amino-4-methoxybenzonitrile (258). To a solution of **250** (685 mg, 3.84 mmol) in ethyl acetate (100 mL) Pd (C) (10 mg) was added and the reaction was stirred at room temperature under H_2 atmosphere for 48 h. By filtration through Celite© and solvent evaporation, 529 mg (93%) of crude reaction **258** was obtained and used without further purification.^{1}H NMR (400 MHz, $CDCl_3$): δ 3.83 (3H, *s*), 6.71 (1H, *d, J* = 8), 6.84 (1H, *d, J* = 2), 6.87 (1H, *s*) 6.98 (1H, *dd, J* = 8 and 2). GC-MS ($C_8H_8N_2O$): calcd 148, found 148.

***N*-(5-cyano-2-methoxyphenyl)-4-methoxybenzenesulfonamide (276A) and 4-cyano-2-((4-methoxyphenyl)sulfonamido)phenyl 4-methoxybenzenesulfonate (276B).** To a solution of **258** (529 mg, 3.57 mmol) in CH_2Cl_2 (50 mL) and pyridine (2 mL) 4-methoxybenzenesulfonyl chloride (1.106 g, 5.35 mmol) was slowly added. The mixture was stirred at room temperature for 4 h. Then the reaction was treated with HCl 2N and $NaHCO_3$ 5%, washed with brine, dried over anhydrous Na_2SO_4 and the solvent evaporated in vacuum to give a residue that was purified by silica gel chromatography using hexane/EtOAc (7:3) to yield the sulfonamides **276A** (466 mg, 41%) and **276B** (205 mg, 30%). **276A:** ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.80 (3H, *s*), 3.83 (3H, *s*), 6.80 (1H, *d, J* = 8), 6.91 (2H, *d, J* = 9.2), 7.09 (1H, *s*), 7.33 (1H, *dd, J* = 8 and 2), 7.75 (2H, *d, J* = 9.2), 7.76 (1H, *d, J* = 2). ^{13}C NMR (100 MHz, $CDCl_3$): δ 55.6 (CH_3), 56.1 (CH_3), 104.5 (C),

110.9 (CH), 114.2 (2CH), 118.6 (C), 122.6 (CH), 127.2 (C), 129.4 (2CH), 129.5 (CH), 130.1 (C), 152.1 (C), 163.4 (C). HRMS ($C_{15}H_{14}N_2O_4S + Na^+$): calcd 341.0569 ($M + Na^+$), found 341.0566. **276B:** IR (KBr): 2233, 1462, 833, 749 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 3.83 (3H, *s*), 3.91 (3H, *s*), 6.91 (2H, *d, J* = 9.2), 7.01 (2H, *d, J* = 8.8), 7.03 (1H, *d, J* = 8), 7.15 (1H, *s*), 7.25 (1H, *dd, J* = 8 and 2), 7.73 (2H, *d, J* = 9.2), 7.75 (2H, *d, J* = 8.8), 7.78 (1H, *d, J* = 2). ^{13}C NMR (100 MHz, $CDCl_3$): δ 55.6 (CH_3), 55.9 (CH_3), 111.5 (C), 114.5 (2CH), 115.0 (2CH), 117.4 (C), 123.7 (CH), 124.4 (CH), 124.8 (C), 128.4 (CH), 129.4 (2CH), 129.8 (C), 130.8 (2CH), 131.5 (C), 142.4 (C), 163.6 (C), 165.1 (C). HRMS ($C_{21}H_{18}N_2O_7S_2 + Na^+$): calcd 497.0448 ($M + Na^+$), found 497.0409.

4-methoxy-3-nitrobenzenesulfonyl chloride (86). To a stirred solution at 0°C of 4-methoxybenzenesulfonyl chloride (4.41 g, 21.36 mmol) in CH_2Cl_2 (20 mL) and H_2SO_4 (5 mL) nitric acid (0.95 mL, 21.36 mmol) was dropwise added under nitrogen atmosphere. After 4 h, the reaction was poured onto ice and the mixture was kept at 4°C for 30 min. Then, the precipitate was filtered under vacuum to dryness to obtain 4.97 g (92%) of **86**. ^1H NMR (400 MHz, $CDCl_3$): δ 4.11 (3H, *s*), 7.33 (1H, *d, J* = 8.8), 8.20 (1H, *dd, J* = 8.8 and 2.4), 8.48 (1H, *d, J* = 2.4). ^{13}C NMR (100 MHz, $CDCl_3$): δ 57.0 (CH_3), 114.0 (CH), 124.8 (CH), 127.1 (C), 132.2 (CH), 135.0 (C), 157.1 (C). GC-MS ($C_7H_6ClNO_5S$): calcd 251, found 251.

***N*-(2,5-dimethoxyphenyl)-4-methoxy-3-nitrobenzenesulfonamide (183).** To 650 mg of the amine **75** (4.24 mmol) in CH_2Cl_2 (50 mL) and pyridine (2 mL), 1.07 g of the sulfonyl chloride **86** was slowly added (4.24 mmol) and stirred at room temperature for 6 h. The reaction was treated with HCl 2N and $NaHCO_3$ 5%, washed with brine, dried over anhydrous Na_2SO_4 and the solvent evaporated to obtain 4.9 g (95%) of **76**. The residue was crystallized CH_2Cl_2/Hexane to afford the purified compound (4.29 g, 84%). M.p.: 121-123 °C (CH_2Cl_2/Hexane). ^1H NMR (400 MHz, $CDCl_3$): δ 3.61 (3H, *s*), 3.72 (3H, *s*), 3.96 (3H, *s*), 6.56 (1H, *dd, J* = 8.8 and 3.2), 6.65 (1H, *d, J* = 8.8), 7.07 (1H, *d, J* = 8.8), 7.09 (1H, *d, J* = 3.2), 7.16 (1H, *s*), 7.88 (1H, *dd, J* = 8.8 and 2.4), 8.22 (1H, *d, J* = 2.4). ^{13}C NMR (100 MHz, $CDCl_3$): δ 55.7 (CH_3), 56.0 (CH_3), 57.0 (CH_3), 108.1 (CH), 110.5 (CH), 111.4 (CH), 113.6 (CH), 125.2 (CH), 125.5 (C), 130.9 (C), 133.1 (CH), 138.8 (C), 143.9 (C), 153.8 (C), 155.8 (C). HRMS ($C_{15}H_{16}N_2O_7S + Na^+$): calcd 391.0563 ($M + Na^+$), found 391.0570.

***N*-(4-bromo-2,5-dimethoxyphenyl)-4-methoxy-*N*-methyl-3-nitrobenzenesulfonamide (204).** To a stirred solution of **183** (86 mg, 0.23 mmol) in CH_2Cl_2 (25 mL) N-bromosuccinimide (41 mg, 0.23 mmol) was added. After 2 h the solvent was evaporated in vacuum and the residue was re-dissolved in CH_3CN. 25 mg of crushed KOH (0.36 mmol) and 17 µL of methyl iodide (0.27 mmol) were added to the reaction mixture and it was stirred at room temperature for 24 h. Finally, it was concentrated, re-dissolved in CH_2Cl_2, washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated in vacuum. The residue was purified by silica gel chromatography using toluene/EtOAc (8:2) to afford 87 mg (80%) of **204**. ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.18 (3H, *s*), 3.43 (3H, *s*), 3.88 (3H, *s*) 4.01 (3H, *s*), 6.96 (1H, *s*), 6.99 (1H, *d, J* = 9.2), 7.01 (1H, *s*), 7.68 (1H, *dd, J* = 9.2 and 2.4), 7.72 (1H, *d, J* = 2.4). ^{13}C NMR (100 MHz, $CDCl_3$): δ 37.7 (CH_3), 55.5 (CH_3), 56.6 (CH_3), 56.9 (CH_3), 111.0 (CH), 112.0 (C), 116.1 (CH), 116.6 (CH), 127.2 (CH), 127.9 (C), 130.2 (CH), 131.6 (C), 139.0 (C), 149.9 (C), 150.0 (C), 157.6 (C). HRMS ($C_{16}H_{17}BrN_2O_7S$ + H^+): calcd 462.9877 (M + H^+), found 462.9992.

***N*-(2,5-dimethoxyphenyl)-4-nitrobenzenesulfonamide (130).** To a solution of **75** (1.06 g, 6.89 mmol) in CH_2Cl_2 (50 mL) and pyridine (2 mL), was slowly added 4-nitrobenzenesulfonyl chloride (1.53 g, 6.89 mmol). The mixture was stirred at room temperature for 4 h. Then the reaction was treated with HCl 2N and $NaHCO_3$ 5%, washed with brine, dried over anhydrous Na_2SO_4 and the solvent evaporated to obtain 2.09 g (90%) of the sulfonamide **130**. The crude reaction product was purified by crystallization in CH_2Cl_2/Hexane (1.274 g, 55%). M.p.: 164-168 °C (CH_2Cl_2/Hexane). ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.57 (3H, *s*), 3.74 (3H, *s*), 6.58 (1H, *dd, J* = 9.2 and 2.8), 6.65 (1H, *d, J* = 9.2), 7.15 (1H, *d, J* = 2.8), 7.93 (2H, *d, J* = 9.2), 8.22 (2H, *d, J* = 9.2). ^{13}C NMR (100 MHz, $CDCl_3$): δ 55.8 (CH_3), 56.0 (CH_3), 108.3 (CH), 110.6 (CH), 111.5 (CH), 123.9 (2CH), 125.3 (C), 128.5 (2CH), 143.8 (C), 144.7 (C), 150.1 (C), 153.8 (C). HRMS ($C_{14}H_{14}N_2O_6S$ + Na^+): calcd 361.0465 (M + Na^+), found 361.0463.

***N*-(4-bromo-2,5-dimethoxyphenyl)-4-nitrobenzenesulfonamide (296).** To a stirred solution of **130** (1.95 g, 5.76 mmol) in CH_2Cl_2 (100 mL) N-bromosuccinimide (1.23 g, 6.92 mmol) was added. After 4 h at room temperature the reaction was washed with water, dried over anhydrous Na_2SO_4 and the solvent evaporated in vacuum to give 2.35 g (98%) of **296.** The crude reaction product was purified by

crystallization in methanol (1.26 g, 52%). M.p.: 190-196 °C (MeOH). ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.57 (3H, *s*), 3.88 (3H, *s*), 6.94 (1H, *s*), 6.97 (1H, *s*), 7.24 (1H, *s*), 7.90 (2H, *d, J* = 8.8), 8.27 (2H, *d, J* = 8.8). ^{13}C NMR (100 MHz, $CDCl_3$): δ 56.8 (CH_3), 57.5 (CH_3), 107.6 (CH), 108.7 (C), 116.6 (CH), 124.6 (2CH), 124.8 (C), 129.0 (2CH), 144.7 (C), 145.1 (C), 150.8 (C), 151.0 (C). HRMS ($C_{14}H_{13}BrN_2O_6S$ + Na^+): calcd 438.9580 and 440.9583 (M + Na^+), found 438.9570 and 440.9549.

4-amino-*N*-(4-bromo-2,5-dimethoxyphenyl)benzenesulfonamide (302). To an EtOH/HOAc/H_2O mixture (2:2:1, 12.5 mL) HCl (c) (1 drop), **296** (2.35 g, 5.63 mmol) and Fe (3.15 g, 56.3 mmol) were added and the reaction stirred for 2 h at 100°C. After extraction with CH_2Cl_2, filtration through celite and treatment with $NaHCO_3$ 5%, the crude reaction mixture was purified by silica gel chromatography using hexane/EtOAc (7:3) to yield 1.02 g (47%) of **302.** IR (KBr): 3368, 1498, 822 cm^{-1}. ^{1}H NMR (400 MHz, CD_3OD): δ 3.55 (3H, *s*), 3.77 (3H, *s*), 6.57 (2H, *d, J* = 8.8), 7.00 (1H, *s*), 7.11 (1H, *s*), 7.39 (2H, *d, J* = 8.8). ^{13}C NMR (100 MHz, Acetone-d_6): δ 56.2 (CH_3), 56.3 (CH_3), 105.0 (C), 105.9 (CH), 112.9 (2CH), 116.3 (CH), 125.6 (C), 127.4 (C), 129.3 (2CH), 144.5 (C), 150.1 (C), 152.9 (C). HRMS ($C_{14}H_{15}BrN_2O_4S$ + Na^+): calcd 408.9828 and 410.9808 (M + Na^+), found 408.9825 and 410.9793.

***N*-(4-(*N*-(4-bromo-2,5-dimethoxyphenyl)sulfamoyl)phenyl)formamide (315).** 690 mg of **302** (1.78 mmol) were dissolved in CH_2Cl_2 (70 mL), pyridine (5 mL) and formic acid (10 mL) and stirred at room temperature. After 24 h, the reaction mixture was poured onto ice and treated with HCl 2N and $NaHCO_3$ 5%. The organic layers were washed to neutrality with brine, dried over anhydrous Na_2SO_4, filtered and evaporated to dryness to afford 708 mg (95%) of **315**. The crude reaction product was purified by crystallization in methanol (307 mg, 41%). M.p.: 202-207 °C (MeOH). IR (KBr): 3337, 1702, 1593, 831 cm^{-1}. ^{1}H NMR (400 MHz, CD_3OD): δ 3.49 (3H, *s*), 3.81 (3H, *s*), 7.01 (1H, *s*), 7.15 (1H, *s*), 7.67 (4H, *bs*), 8.30 (1H, *s*). ^{13}C NMR (100 MHz, DMSO-d_6): δ 56.9 (CH_3), 57.0 (CH_3), 107.1 (C), 109.8 (CH), 117.1 (CH), 119.1 (2CH), 126.2 (C), 128.6 (2CH), 134.8 (C), 142.8 (C), 146.9 (C), 149.6 (C), 160.6 (CH). HRMS ($C_{15}H_{14}BrN_2O_5S$ + Na^+): calcd 436.9777 and 438.9757 (M + Na^+), found 436.9772 and 438.9750.

***N*-(4-bromo-2,5-dimethoxyphenyl)-4-(methylamino)benzenesulfonamide (323).** To a solution of the formamide **315** (680 mg, 1.64 mmol) and $NaBH_4$ (93 mg, 2.45 mmol) in dry THF (15 mL) at 0°C, trichloroacetic acid (401 mg, 2.45 mmol) in dry THF (10 mL) was added dropwise under nitrogen atmosphere. The reaction mixture was stirred at 0°C to room temperature for 24 h and then concentrated and re-dissolved in EtOAc, washed with brine, dried over anhydrous Na_2SO_4, filtered and solvent evaporated in vacuum. The residue was purified by silica gel chromatography using hexane/EtOAc (8:2) to yield 246 mg (37%) of **323**. IR (KBr): 3420, 3251, 1599, 820 cm^{-1}. ^1H NMR (400 MHz, CD_3OD): δ 2.76 (3H, *s*), 3.56 (3H, *s*), 3,78 (3H, *s*), 6.51 (2H, *d, J* = 8.8), 7.00 (1H, *s*), 7.13 (1H, *s*), 7,45 (2H, *d, J* = 8.8). ^{13}C NMR (100 MHz, $CDCl_3$): δ 30.0 (CH_3), 56.5 (CH_3), 56.8 (CH_3), 105.3 (CH), 105.5 (C), 111.1 (2CH), 115.8 (CH), 125.0 (C), 126.6 (C), 129.3 (2CH), 143.5 (C), 150.2 (C), 152.7 (C). HRMS ($C_{15}H_{16}BrN_2O_4S + Na^+$): calcd 422.9985 and 424.9964 ($M + Na^+$), found 422.9985 and 424.9959.

***N*-benzyl-*N*-(4-bromo-2,5-dimethoxyphenyl)-4-(methylamino)benzenesulfonamide (332).** 35 mg (0.25 mmol) of K_2CO_3 were added to a stirred solution of **323** (50 mg, 0.12 mmol) in 3 mL of dry DMF. After 1 h at room temperature 21.7 µL (0.19 mmol) of benzyl chloride were added and stirred for 24 h. The reaction mixture was concentrated, re-dissolved in CH_2Cl_2, washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated in vacuum to obtain 59 mg (96%) and crystalized in MeOH (23 mg, 38%). M.p.: 177-183 °C (MeOH). ^1H NMR (400 MHz, $CDCl_3$): δ 2.79 (3H, *s*), 3.33 (3H, *s*), 3.57 (3H, *s*), 4.64 (2H, *s*), 6.46 (2H, *d, J* = 8.8), 6.52 (1H, *s*), 6.85 (1H, *s*), 7.14 (5H, *m*), 7.44 (2H, *d, J* = 8.8). ^{13}C NMR (100 MHz, $CDCl_3$): δ 30.1 (CH_3), 53.3 (CH_2), 55.7 (CH_3), 56.7 (CH_3), 110.8 (2CH), 111.49 (C), 116.5 (CH), 117.5 (CH), 126.5 (C), 126.9 (C), 127.4 (CH), 128.2 (2CH), 128.7 (2CH), 129.7 (2CH), 136.7 (C), 149.4 (C), 151.0 (C), 152.4 (C). HRMS ($C_{22}H_{23}BrN_2O_4S + Na^+$): calcd 515.0415 ($M + Na^+$), found 515.0434.

3,4,5-trimethoxy-2-((4-methoxyphenyl)sulfonamido)benzoic acid (63A) and 3,4,5-trimethoxy-2-(3,4,5-trimethoxy-2-((4-methoxyphenyl)sulfonamido)benzamido) benzoic acid (63B) To a stirred solution of 2-amino-3,4,5-trimethoxybenzoic acid (300 mg, 1.32 mmol) in CH_2Cl_2 (50 mL) and pyridine (2 mL) 4-methoxybenzenesulfonyl chloride (273 g, 1.32 mmol) was slowly added. After 6 h, the reaction mixture was poured into HCl 2N solution and extracted with CH_2Cl_2. The organic layers were washed to

neutrality with saturated NaCl, dried over anhydrous Na_2SO_4, filtered and evaporated to dryness. The residue was purified by two successive crystallizations in CH_2Cl_2/hexane, compounds **63A** (40 mg, 8 %) and **63B** (17 mg, 4 %) were isolated. **63A:** M.p.: 165-167 °C (CH_2Cl_2/Hexane). IR (KBr):3264, 2939, 1668, 1458, 837 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 3.45 (3H, *s*), 3.84 (3H, *s*), 3.88 (3H, *s*), 3.91 (3H, *s*), 6.93 (2H, *d, J* = 8.8), 7.28 (1H, *s*), 7.77 (2H, *d, J* = 8.8), 8.7 (1H, *s*). ^{13}C NMR (100 MHz, $CDCl_3$): δ 55.6 (CH_3), 56.1 (CH_3), 60.3 (CH_3), 61.1 (CH_3), 109.0 (CH), 113.7 (2CH), 116.5 (C), 128.0 (C), 129.4 (2CH), 132.0 (C), 147.7 (C), 148.4 (C), 150.5 (C), 162.8 (C), 171.4 (C). HRMS ($C_{17}H_{19}NO_8S + H^+$): calcd 398.0901 ($M + H^+$), found 398.0905. **63B:** M.p.: 170-171 °C (CH_2Cl_2/Hexane). ^1H NMR (400 MHz, $CDCl_3$): δ 3.35 (3H, *s*), 3.75 (3H, *s*), 3.79 (3H, *s*), 3.84 (3H, *s*), 3.88 (3H, *s*), 3.96 (3H, *s*), 3.99 (3H, *s*), 6.85 (2H, *d, J* = 8.8), 7.20 (1H, *s*), 7.25 (1H, *s*), 7.68 (2H, *d, J* = 8.8), 8.15 (1H, *s*), 9,22 (1H, *s*). ^{13}C NMR (100 MHz, $CDCl_3$): δ 57.1 (CH_3), 59.0 ($2CH_3$), 61.9 (CH_3), 62.4 (CH_3), 62.6 (CH_3), 63.0 (CH_3), 108.9 (CH), 110.2 (CH), 115.2 (2CH), 120.9 (C), 124.1 (C), 126.6 (C), 127.4 (C), 131.3 (2CH), 133.2 (C), 147.0 (C), 148.3 (C), 149.7 (C), 149.9 (C), 152.9 (C), 153.4 (C), 153.9 (C), 165.3 (C), 167.5 (C). HRMS ($C_{27}H_{30}N_2O_{12}S + H^+$): calcd 607.1599 ($M + H^+$), found 607.1593.

***N*-(3,5-dimethoxyphenyl)-4-methoxybenzenesulfonamide (259).** To a solution of 3,5-dimethoxyaniline (290 mg, 1.89 mmol) in CH_2Cl_2 (50 mL) and pyridine (2 mL), was slowly added 4-methoxybenzenesulfonyl chloride (469 mg, 2.27 mmol). The mixture was stirred at room temperature for 4 h. Then the reaction was treated with HCl 2N and $NaHCO_3$ 5%, washed with brine, dried over anhydrous Na_2SO_4 and the solvent evaporated to obtain 596 mg (97%) of the sulfonamide **259**. The crude reaction product was purified by crystallization in CH_2Cl_2/Hexane (438 mg, 71%). M.p.: 115-122 °C (CH_2Cl_2/Hexane). IR (KBr): 3234, 1595, 824 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 3.66 (6H, *s*), 3.77 (3H, *s*), 6.13 (1H, *t, J* = 2), 6.17 (2H, *d, J* = 2), 6.85 (2H, *d, J* = 8.8), 7.68 (2H, *d, J* = 8.8). ^{13}C NMR (100 MHz, $CDCl_3$): δ 55.3 ($2CH_3$), 55.5 (CH_3), 97.0 (CH), 98.9 (2CH), 114.2 (2CH), 129.5 (2CH), 130.4 (C), 138.6 (C), 161.1 (2C), 163.1 (C). HRMS ($C_{15}H_{17}NO_5S + H^+$): calcd 324.0909 ($M + H^+$), found 324.0900.

***N*-benzyl-*N*-(3,5-dimethoxyphenyl)-4-methoxybenzenesulfonamide (270).** To a stirred solution of **259** (90 mg, 0.28 mmol) in dry DMF (3 mL) 78 mg (0.56 mmol) of K_2CO_3 were added. After 1 h at room temperature, 48.5 µL (0.42 mmol) of benzyl chloride were added and stirred for 24 h. The reaction mixture

was concentrated, re-dissolved in CH_2Cl_2, washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated under vacuum to produced 104 mg (90%) of crude reaction product from which 81 mg (70%) of **270** were purified by crystallization. M.p.: 146-150 °C (MeOH). IR (KBr): 3467, 1458, 806 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 3.62 (6H, *s*), 3.87 (3H, *s*), 4.65 (2H, *s*), 6.12 (2H, *d, J* = 2), 6.28 (1H, *t, J* = 2), 6.94 (2H, *d, J* = 8.8), 7.22 (5H, *m*), 7.63 (2H, *d, J* = 8.8). ^{13}C NMR (100 MHz, $CDCl_3$): δ 54.7 (CH_2), 55.3 ($2CH_3$), 55.6 (CH_3), 100.0 (CH), 107.2 (2CH), 113.9 (2CH), 127.5 (CH), 128.3 (2CH), 128.5 (2CH), 129.8 (2CH), 130.3 (C), 136.1 (C), 140.9 (C), 160.4 (2C), 162.9 (C). HRMS ($C_{22}H_{23}NO_5S + H^+$): calcd 414.1370 ($M + H^+$), found 414.1369.

***N*-benzyl-*N*-(4-bromo-3,5-dimethoxyphenyl)-4-methoxybenzenesulfonamide (326A), *N*-benzyl-*N*-(2-bromo-3,5-dimethoxyphenyl)-4-methoxybenzenesulfonamide (326B) and *N*-benzyl-*N*-(2,4-dibromo-3,5-dimethoxyphenyl)-4-methoxybenzenesulfonamide (326C).** To a solution of **270** (195 mg, 0.47 mmol) in CH_2Cl_2 (40 mL) N-bromosuccinimide (168 mg, 0.94 mmol) was added and stirred for 48 h at room temperature. After that, the reaction was washed with water, dried over anhydrous Na_2SO_4 and the solvent evaporated in vacuum to produce 183 mg of crude reaction. The residue was flash chromatographed on silica gel (hexane/EtOAc 8:2) to afford the purified compounds: **326A** (116 mg, 50%), **326B** (24 mg, 10%) and **326C** (6 mg, 2%). **326A:** M.p.: 199-203 °C (MeOH). IR (KBr): 3435, 1589, 836 cm^{-1}. ^1H NMR (200 MHz, $CDCl_3$): δ 3.65 (6H, *s*), 3.88 (3H, *s*), 4.68 (2H, *s*), 6.12 (2H, *s*), 6.97 (2H, *d, J* = 9), 7.22 (5H, *bs*), 7.65 (2H, *d, J* = 9). ^{13}C NMR (100 MHz, $CDCl_3$): δ 54.9 (CH_2), 55.7 (CH_3), 56.4 ($2CH_3$), 100.5 (C), 105.8 (2CH), 114.0 (2CH), 127.8 (CH), 128.4 (2CH), 128.6 (2CH), 129.9 (C), 130.0 (2CH), 135.7 (C), 139.4 (C), 156.7 (2C), 163.1 (C). HRMS ($C_{22}H_{22}BrNO_5S + H^+$): calcd 492.0475 and 494.0454 ($M + H^+$), found 492.0473 and 494.0440. **326B:** IR (KBr): 2938, 1593, 831 cm^{-1}. ^1H NMR (200 MHz, $CDCl_3$): δ 3.57 (3H, *s*), 3.80 (3H, *s*), 3.87 (3H, *s*), 4.60 (1H, *d, J* = 14.4), 4.89 (1H, *d, J* = 14.4), 6.14 (1H, *d, J* = 2.8), 6.38 (1H, *d, J* = 2.8), 6.94 (2H, *d, J* = 9) 7.20 (5H, *bs*), 7.74 (2H, *d, J* = 9). ^{13}C NMR (100 MHz, $CDCl_3$): δ 54.4 (CH_2), 55.5 (CH_3), 55.6 (CH_3), 56.3 (CH_3), 100.0 (CH), 105.9 (C), 109.4 (CH), 113.9 (2CH), 127.7 (CH), 128.2 (2CH), 129.4 (2CH), 130.1 (2CH), 131.8 (C), 135.7 (C), 138.8 (C), 157.2 (C), 158.9 (C), 163.0 (C). HRMS ($C_{22}H_{22}BrNO_5S + H^+$): calcd 492.0475 and 494.0454 ($M + H^+$), found 492.0467 and 494.0447. **326C:** IR (KBr): 2935, 1595, 835 cm^{-1}. ^1H NMR (200 MHz, $CDCl_3$): δ 3.60 (3H, *s*), 3.77 (3H, *s*), 3.87 (3H, *s*), 4.55 (1H, *d, J* = 14.4), 4.97 (1H, *d, J* = 14.4), 6.30 (1H, *s*), 6.95 (2H, *d, J* = 9), 7.22

(5H, *m*), 7.73 (2H, *d, J* = 9). ^{13}C NMR (100 MHz, $CDCl_3$): δ 54.2 (CH_2), 55.6 (CH_3), 56.5 (CH_3), 60.5 (CH_3), 108.8 (C), 112.4 (C), 112.8 (CH), 114.0 (2CH), 128.0 (CH), 128.3 (2CH), 129.4 (2CH), 130.0 (2CH), 131.5 (C), 135.4 (C), 137.4 (C), 155.6 (C), 155.7 (C), 163.2 (C). HRMS ($C_{22}H_{21}Br_2NO_5S$ + H): calcd 569.9580 and 571.9559 (M^+ + H), found 569.9577 and 571.9558.

***N*-(3,5-dimethoxyphenyl)-4-nitrobenzenesulfonamide (242).** To a solution of 3,5-dimethoxyaniline (1.39 g, 9.98 mmol) in CH_2Cl_2 (50 mL) and pyridine (2 mL), was slowly added 4-nitrobenzenesulfonyl chloride (2.21 g, 9.07 mmol) and stirred at room temperature for 12 h. Then the mixture reaction was treated with HCl 2N and $NaHCO_3$ 5%. The organic layers were washed to neutrality with brine, dried over anhydrous Na_2SO_4 and concentrated under vacuum to yield 2.66 g (87%) of the sulfonamide **242**. The crude reaction product was purified by crystallization in CH_2Cl_2/Hexane (2.43 g, 79%). M.p.: 131-139 °C (CH_2Cl_2/Hexane). ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.71 (6H, *s*), 6.22 (1H, *t, J* = 2.4), 6.25 (2H, *d, J* = 2.4), 7.98 (2H, *d, J* = 8.8), 8.28 (2H, *d, J* = 8.8). ^{13}C NMR (100 MHz, $CDCl_3$): δ 55.4 ($2CH_3$), 97.4 (CH), 99.7 (2CH), 124.3 (2CH), 128.6 (2CH), 137.4 (C), 144.4 (C), 150.1 (C), 161.3 (2C). HRMS ($C_{14}H_{14}N_2O_6S$ + H^+): calcd 339.0653 (M + H^+), found 339.0645.

4-amino-*N*-(3,5-dimethoxyphenyl)benzenesulfonamide (245). To a solution of **242** (2.60 g, 7.69 mmol) in ethyl acetate (150 mL) an MeOH (5 mL), Pd (C) (10 mg) was added and the reaction was stirred at room temperature under H_2 atmosphere for 24 h. By filtration through celite and solvent evaporation, 2.30 g (97%) of **245** was obtained and purified by crystallization in MeOH (1.53 g, 65%). M.p.: 149-155 °C (MeOH). IR (KBr): 3450, 3370, 1458, 821 cm^{-1}. ^{1}H NMR (400 MHz, CD_3OD): δ 3.67 (6H, *s*), 6.13 (1H, *t, J* = 2.4), 6.25 (2H, *d, J* = 2.4), 6.60 (2H, *d, J* = 8.8), 7.46 (2H, *d, J* = 8.8). ^{13}C NMR (100 MHz, CD_3OD): δ 54.2 ($2CH_3$), 95.6 (CH), 98.2 (2CH), 112.8 (2CH), 125.3 (C), 128.8 (2CH), 139.8 (C), 152.8 (C), 161.1 (2C). HRMS ($C_{14}H_{16}N_2O_4S$ + H^+): calcd 309.0904 (M + H^+), found 309.0915.

***N*-(3,5-dimethoxyphenyl)-4-(dimethylamino)benzenesulfonamide (254).** To a solution of p-formaldehyde (534 mg, 17.77 mmol) in MeOH (40 mL), 548 mg of **245** (1.77 mmol) were added and stirred for 30 min, then $NaCNBH_3$ (223 mg, 10.62 mmol) was added and the reaction was heated at reflux for 24 h.

The reaction mixture was concentrated, poured onto ice and extracted with EtOAc, dried over Na_2SO_4, filtered through celite and the solvent evaporated in vacuum to afford 580 mg (97%) of **254**. M.p.: 158-164 °C (MeOH). IR (KBr): 3228, 1498, 812 cm^{-1}. ^1H NMR (400 MHz, CD_3OD): δ 2.99 (6H, *s*), 3.67 (6H, *s*), 6.12 (1H, *t, J* = 2.4), 6.26 (2H, *d, J* = 2.4), 6.68 (2H, *d, J* = 9.2), 7.58 (2H, *d, J* = 9.2). ^{13}C NMR (100 MHz, $CDCl_3$): δ 39.9 ($2CH_3$), 55.3 ($2CH_3$), 96.6 (CH), 98.4 (2CH), 110.8 (2CH), 123.9 (C), 129.1 (2CH), 139.1 (C), 152.9 (C), 161.1 (2C). HRMS ($C_{16}H_{20}N_2O_4S + H^+$): calcd 337.1217 ($M + H^+$), found 337.1205.

***N*-benzyl-*N*-(3,5-dimethoxyphenyl)-4-(dimethylamino)benzenesulfonamide (275).** 77 mg (0.55 mmol) of K_2CO_3 were added to a stirred solution of **254** (93 mg, 0.27 mmol) in 3 mL of dry DMF. After 1 h at room temperature 48.2 µL (0.41 mmol) of benzyl chloride were added and stirred for 24 h. The reaction mixture was concentrated, re-dissolved in EtOAc, washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated in vacuum to obtain 112 mg (95%) and crystalized in MeOH (62 mg, 52%). M.p.: 155-160 °C (MeOH). IR (KBr): 3471, 1455, 811cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 3.05 (6H, *s*), 3.63 (6H, *s*), 4.63 (2H, *s*), 6.18 (2H, *d, J* = 2.4), 6.26 (1H, t, J = 2.4), 6.64 (2H, *d, J* = 8.8), 7.20 (5H, *m*), 7.53 (2H, *d, J* = 8.8). ^{13}C NMR (100 MHz, $CDCl_3$): δ 40.1 ($2CH_3$), 54.5 (CH_2), 55.3 ($2CH_3$), 99.9 (CH), 107.1 (2CH), 110.6 (2CH), 123.9 (C), 127.4 (CH), 128.2 (2CH), 128.5 (2CH), 129.6 (2CH), 136.4 (C), 141.4 (C), 152.8 (C), 160.3 (2C). HRMS ($C_{23}H_{26}N_2O_4S + H^+$): calcd 427.1686 ($M + H^+$), found 427.1658.

***N*-(6-methoxypyridin-3-yl)-4-nitrobenzenesulfonamide (283).** To a solution of 6-methoxypyridin-3-amine (1.82 g, 14.66 mmol) in CH_2Cl_2 (50 mL) and pyridine (2 mL), was slowly added 4-nitrobenzenesulfonyl chloride (3.9 g, 17.59 mmol). The mixture was stirred at room temperature for 4 h. Then the reaction was treated with HCl 2N and $NaHCO_3$ 5%, washed with brine, dried over anhydrous Na_2SO_4 and the solvent evaporated to obtain 3.10 g (68%) of **273**. It was purified by crystallization in MeOH 2.67 g (59%). M.p.: 139-143 °C (MeOH). IR (KBr): 3208, 1610, 1350, 826 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 3.88 (3H, *s*), 6.49 (1H, *s*), 6.70 (1H, *d, J* = 8.8), 7.43 (1H, *dd, J* = 8.8 and 2.8), 7.72 (1H, *d, J* = 2.8), 7.88 (2H, *d, J* = 9.2), 8.31 (2H, *d, J* = 9.2). ^{13}C NMR (100 MHz, $CDCl_3$): δ 53.8 (CH_3), 111.5 (CH), 124.4 (2CH), 125.2 (C), 128.6 (2CH), 136.3 (CH), 143.2 (CH), 144.3 (C), 150.3 (C), 163.1 (C). HRMS ($C_{12}H_{11}N_3O_5S + H^+$): calcd 310.0492 ($M + H^+$), found 310.0487.

4-amino-*N*-(6-methoxypyridin-3-yl)benzenesulfonamide (287). 3.00 g of **283** (9.71 mmol) was suspended in ethyl acetate (120 mL) and was palladium-catalyzed (Pd (C) 10 mg) reduced under H_2 atmosphere for 72 h. The reaction mixture was filtered through celite and the solvent evaporated in vacuum to isolate 2.67 g (98%) of **287**. Crude reaction product was purified by crystallization in MeOH (539 mg, 20%). M.p.: 176-180 °C (MeOH). IR (KBr): 3485, 3281, 1619, 1500, 794 cm^{-1}. ^1H NMR (400 MHz, CD_3OD): δ 3.82 (3H, *s*), 6.59 (2H, *d, J* = 8.4), 6.68 (1H, *d, J* = 8.8), 7.33 (2H, *d, J* = 8.4), 7.41 (1H, *dd, J* = 8.8 and 2.4), 7.71 (1H, *d, J* = 2.4). ^{13}C NMR (100 MHz, CD_3OD): δ 52.7 (CH_3), 110.1 (CH), 112.8 (2CH), 124.6 (C), 128.4 (C), 128.8 (2CH), 135.2 (CH), 141.2 (CH), 152.9 (C), 161.9 (C). HRMS ($C_{12}H_{13}N_3O_5S + H^+$): calcd 280.0750 ($M + H^+$), found 280.0745.

***N*-(4-(*N*-(6-methoxypyridin-3-yl)sulfamoyl)phenyl)formamide (309).** 970 mg of **287** (3.57 mmol) were dissolved in CH_2Cl_2 (100 mL), pyridine (5 mL) and formic acid (15 mL) and stirred at room temperature. After 24 h, the reaction mixture was poured onto ice and treated with HCl 2N and $NaHCO_3$ 5%. The organic layers were washed to neutrality with brine, dried over anhydrous Na_2SO_4, filtered and evaporated to dryness to afford 555 mg (52%) of **309**. The crude reaction product was purified by crystallization in methanol (368 mg, 34%). M.p.: 189-193 °C (MeOH). IR (KBr): 3349, 3273, 1698, 1592, 823 cm^{-1}. ^1H NMR (400 MHz, CD_3OD): δ 3.82 (3H, *s*), 6.69 (1H, *d, J* = 8.8), 7.42 (1H, *dd, J* = 8.8 and 2.8), 7.62 (2H, *d, J* = 8.8), 7.70 (2H, *d, J* = 8.8), 7.71 (1H, *d, J* = 2.8), 8.31 (1H, *s*). ^{13}C NMR (100 MHz, Acetone-d6): δ 52.8 (CH_3), 110.6 (CH), 118.9 (2CH), 128.0 (C), 128.4 (2CH), 133.9 (C), 134.8 (CH), 141.6 (CH), 142.3 (C), 159.6 (C), 161.9 (CH). HRMS ($C_{13}H_{13}N_3O_4S + H^+$): calcd 308.0703 ($M + H^+$), found 308.0700.

***N*-(6-methoxypyridin-3-yl)-4-(methylamino)benzenesulfonamide (316).** To a solution of the formaldehyde **309** (485 mg, 1.58 mmol) and $NaBH_4$ (90 mg, 2.37 mmol) in dry THF (15 mL) at 0°C, trichloroacetic acid (387 mg, 2.37 mmol) in dry THF (10 mL) was added dropwise under nitrogen atmosphere. The reaction mixture was stirred at 0°C to room temperature for 24 h and then concentrated and re-dissolved in EtOAc, washed with brine, dried over anhydrous Na_2SO_4, filtered and solvent evaporated in vacuum. The crude reaction product (334 mg, 72%) was purified by crystallization in MeOH to yield 137 mg (30%) of **316**. M.p.: 183-186 °C (MeOH). IR (KBr): 3387, 3277, 1500, 821 cm^{-1}. ^1H NMR (400

MHz, CD_3OD): δ 2.76 (3H, *s*), 3.82 (3H, *s*), 6.52 (2H, *d, J* = 8.8), 6.67 (1H, *d, J* = 8.8), 7.38 (2H, *d, J* = 8.8), 7.41 (1H, *dd, J* = 8.8 and 2.8), 7.71 (1H, *d, J* = 2.8). ^{13}C NMR (100 MHz, $CDCl_3$): δ 30.0 (CH_3), 53.6 (CH_3), 111.0 (CH), 111.3 (2CH), 124.8 (C), 127.0 (C), 129.3 (2CH), 135.9 (CH), 142.5 (CH), 152.7 (C), 162.4 (C). HRMS ($C_{13}H_{15}N_3O_3S + H^+$): calcd 294.0907 ($M + H^+$), found 294.0907.

***N*-(4-(*N*-(6-methoxypyridin-3-yl)sulfamoyl)phenyl)-*N*-methylformamide (320).** The compound **316** (88 mg, 0.30 mmol) was stirred in a mixture of CH_2Cl_2 (50 mL), pyridine (2 mL) and formic acid (5 mL) at room temperature for 24 h. The reaction mixture was poured onto ice and treated with HCl 2N and $NaHCO_3$ 5%, washed to neutrality with brine, dried over anhydrous Na_2SO_4, filtered and solvent evaporated to produce 33 mg (34%) of **320**, which crystallized in methanol (24 mg, 25%). M.p.: 164-172 °C (MeOH). ^1H NMR (400 MHz, CD_3OD): δ 3.30 (3H, *s*), 3.82 (3H, *s*), 6.69 (1H, *d, J* = 8.8), 7.44 (2H, *d, J* = 8.8), 7.46 (1H, *dd, J* = 8.8 and 2.8), 7.71 (1H, *d, J* = 2.8), 7.74 (2H, *d, J* = 8.8), 8.65 (1H, *s*). ^{13}C NMR (100 MHz, CD_3OD): δ 29.9 (CH_3), 52.8 (CH_3), 110.7 (CH), 120.2 (2CH), 127.9 (C), 128.6 (2CH), 134.8 (CH), 135.6 (C), 141.5 (CH), 146.3 (C), 161.4 (C), 161.9 (CH). HRMS ($C_{14}H_{15}N_3O_4S + H^+$): calcd 322.0856 ($M + H^+$), found 322.0855.

4-methoxy-*N*-(6-methoxypyridin-3-yl)benzenesulfonamide (240). To a stirred solution of 6-methoxypyridin-3-amine (312 mg, 2.51 mmol) in CH_2Cl_2 (25 mL) and pyridine (1 mL) 4-methoxybenzenesulfonyl chloride (623 mg, 3.02 mmol) was added under nitrogen atmosphere. After 6 h, the reaction mixture was treated with HCl 2N and $NaHCO_3$ 5% solutions. The organic layers were washed to neutrality with saturated NaCl, dried over anhydrous Na_2SO_4, filtered and evaporated to yield 631 mg (85%) of the sulfonamide **240**. The residue was purified by flash chromatography on silica gel using hexane/EtOAc (6:4) to afford 464 mg (62%). ^1H NMR (400 MHz, $CDCl_3$): δ 3.80 (3H, *s*), 3.83 (3H, *s*), 6.62 (1H, *d, J* = 8.8), 6.87 (2H, *d, J* = 8.8), 7.40 (1H, *dd, J* = 8.8 and 2.8), 7.63 (2H, *d, J* = 8.8), 7.75 (1H, *d, J* = 2.8). ^{13}C NMR (100 MHz, $CDCl_3$): δ 54.3 (CH_3), 56.2 (CH_3), 111.6 (CH) 114.9 (2CH), 127.5 (C), 130.1 (2CH), 130.6 (C), 136.3 (CH), 143.1 (CH), 162.9 (C), 163.8 (C). HRMS ($C_{13}H_{14}N_2O_4S + H^+$): calcd 295.0747 ($M + H^+$), found 295.0761.

***N*-benzyl-4-methoxy-*N*-(6-methoxypyridin-3-yl)benzenesulfonamide (279).** 92 mg (0.31 mmol) of **240** dissolved in dry DMF (3 mL) were stirred for 1 h in the presence of K_2CO_3 (97 mg, 0.62 mmol). After

that, benzyl chloride (54.5 µL 0.47 mmol) was added and stirred for 24 h. The reaction mixture was concentrated, re-dissolved in EtOAc, washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated in vacuum to obtain 112 mg (93%) and crystalized in MeOH (77 mg, 64%). M.p.: 144-146 °C (MeOH). IR (KBr): 3435, 1490, 823 cm^{-1}. ^1H NMR (400 MHz, CD_3OD): δ 3.81 (3H, *s*), 3.89 (3H, *s*), 4.72 (2H, *s*), 6.61 (1H, *d, J* = 8.8), 7.10 (2H, *d, J* = 8.8), 7.22 (5H, *bs*), 7.23 (1H, *dd, J* = 8.8 and 2.8), 7.62 (2H, *d, J* = 8.8), 7.67 (1H, *d, J* = 2.8). ^{13}C NMR (100 MHz, $CDCl_3$): δ 53.7 (CH_3), 54.9 (CH_2), 55.7 (CH_3), 110.8 (CH), 114.2 (2CH), 127.8 (CH), 128.5 (2CH), 128.6 (2CH), 129.2 (C), 129.7 (2CH), 130.7 (C), 135.4 (C), 139.5 (CH), 147.2 (CH), 163.0 (C), 163.1 (C). HRMS ($C_{20}H_{20}N_2O_4S + H^+$): calcd 385.1217 ($M + H^+$), found 385.1210.

***N*-(3,4-dimethoxyphenyl)-4-methoxybenzenesulfonamide (8).** To 2.49 g of 3,4-dimethoxyaniline (16.26 mmol) in CH_2Cl_2 (50 mL) and pyridine (4 mL), 3.61 g of 4-methoxybenzenesulfonyl chloride was slowly added (16.26 mmol) and stirred at room temperature for 12 h. The reaction was treated with HCl 2N and $NaHCO_3$ 5%, washed with brine, dried over anhydrous Na_2SO_4 and the solvent evaporated to obtain 5.29 g (99%) of **8**. The residue was crystallized in CH_2Cl_2/Hexane to afford the purified compound (4.04 g, 75%). M.p.: 101-102 °C (CH_2Cl_2/Hexane). IR (KBr): 3224, 1498, 801 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 3.75 (3H, *s*), 3.79 (3H, *s*), 3.80 (3H, *s*), 6.53 (1H, *dd, J* = 8.8 and 2.8), 6.66 (1H, *d, J* = 8.8), 6.70 (1H, *d, J* = 2.8), 6.86 (2H, *d, J* = 8.8), 7.66 (2H, *d, J* = 8.8). ^{13}C NMR (100 MHz, $CDCl_3$): δ 55.5 (CH_3), 55.9 (CH_3), 55.9(CH_3), 107.7 (CH), 111.1 (CH), 114.0 (2CH), 115.4 (CH), 129.4 (2CH), 129.5 (C), 130.3 (C), 147.2 (C), 149.1 (C), 163.0 (C). HRMS ($C_{15}H_{17}NO_5S + H^+$): calcd 324.0900 ($M + H^+$), found 324.0906.

***N*-(4,5-dimethoxy-2-nitrophenyl)-4-methoxybenzenesulfonamide (11).** To a solution of **8** (588 mg, 1.82 mmol) in CH_3CN (50 mL) tertbutyl nitrite (120 µL, 0.91 mmol) was added and stirred at 45°C. After 1 h, additional 0.91 mmol tertbutyl nitrite was added to the reaction mixture and it was stirred at 45°C for 24 h. The mixture was poured into ice and basified with 5% $NaHCO_3$ solution and extracted with EtOAc. The organic layers were washed with brine, dried over Na_2SO_4 and concentrated under vacuum to yield **11** (638 mg, 95%). The residue was purified by crystallization in CH_2Cl_2/Hexane to afford 527 mg (78%). M.p.: 152-154 °C (CH_2Cl_2/Hexane). IR (KBr): 3257, 1521, 1499, 804 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 3.83 (3H, *s*), 3.87 (3H, *s*), 3.98 (3H, *s*), 6.90 (2H, *d, J* = 9.2), 7.35 (1H, *s*), 7.53 (1H, *s*), 7.23 (2H, *d, J* = 9.2). ^{13}C NMR (100 MHz,

497 $CDCl_3$): δ 55.7 (CH_3), 56.3 (CH_3), 56.7 (CH_3), 103.0 (CH), 107.2 (CH), 114.4 (2CH), 129.4 (2CH), 129.7 (C),
498 129.9 (C), 130.3 (C), 145.2 (C), 155.4 (C), 163.6 (C). HRMS ($C_{15}H_{16}N_2O_7S + H^+$): calcd 369.0751 ($M + H^+$), found
499 369.0752.

500 ***N*-(2-amino-4,5-dimethoxyphenyl)-4-methoxybenzenesulfonamide (12).** To a solution of **11** (620
501 mg, 1.68 mmol) in ethyl acetate (100 mL) Pd (C) (10 mg) was added and the reaction was stirred at room
502 temperature under H_2 atmosphere for 48 h. By filtration through celite and solvent evaporation, 550 mg
503 (96%) of crude reaction **12** was obtained. 374 mg (65%) of the purified compound were isolated by
504 crystallization in CH_2Cl_2/Hexane. M.p.: 102-112 °C (CH_2Cl_2/Hexane). IR (KBr): 3265, 1458, 835 cm^{-1}. ^1H NMR
505 (400 MHz, $CDCl_3$): δ 3.46 (3H, *s*), 3.74 (3H, *s*), 3.79 (3H, *s*), 5.72 (1H, *s*), 5.88 (1H, *s*), 6.23 (1H, *s*), 6.87 (2H, *d,*
506 *J* = 8.4), 7.61 (2H, *d, J* = 8.4). ^{13}C NMR (100 MHz, $CDCl_3$): δ 55.6 (CH_3), 55.8 (CH_3), 56.2 (CH_3), 101.0 (CH),
507 112.4 (C), 112.9 (CH), 114.1 (2CH), 129.8 (2CH), 130.4 (C), 139.0 (C), 141.5 (C), 149.6 (C), 163.1 (C). HRMS
508 ($C_{15}H_{18}N_2O_5S + H^+$): calcd 339.1009 ($M + H^+$), found 339.1018.

509 ***N*-(2-amino-4,5-dimethoxyphenyl)-4-methoxy-*N*-methylbenzenesulfonamide (120).** To a solution
510 of **12** (167 mg, 0.49 mmol) in CH_3CN (25 mL) 68 mg of crushed KOH (0.98 mmol) and 62 µL of methyl iodide
511 (0.98 mmol) were added and stirred at room temperature for 24 h. Then, the reaction mixture was
512 concentrated, re-dissolved in CH_2Cl_2, washed with brine, dried over anhydrous Na_2SO_4, filtered and
513 concentrated in vacuum to produce 140 mg (80%) of **120**. The crude reaction product was purified by
514 crystallization in MeOH (52 mg, 30%). M.p.: 140-141 °C (MeOH). ^1H NMR (400 MHz, $CDCl_3$): δ 3.11 (3H, *s*),
515 3.46 (3H, *s*), 3.82 (3H, *s*), 3.87 (3H, *s*), 5.80 (1H, *s*), 6.34 (1H, *s*), 6.97 (2H, *d, J* = 8.8), 7.65 (2H, *d, J* = 8.8). ^{13}C
516 NMR (100 MHz, $CDCl_3$): δ 40.2 (CH_3), 56.9 (CH_3), 57.0 (CH_3), 57.6 (CH_3), 101.9 (CH), 112.1 (CH), 115.2 (2CH),
517 119.4 (C), 129.8 (C), 131.6 (2CH), 141.5 (C), 142.2 (C), 151.1 (C), 164.4 (C). HRMS ($C_{16}H_{20}N_2O_5S + H^+$): calcd
518 353.1166 ($M + H^+$), found 353.1179.

519 ***N*-(4,5-dimethoxy-2-((4-methoxy-*N*-methylphenyl)sulfonamido)phenyl)acetamide (124).** 90 mg of
520 **120** (0.25 mmol) were dissolved in CH_2Cl_2 (45 mL) and pyridine (1 mL). 29 µL of anhydride acetic acid (0.30
521 mmol) were added to the solution and stirred at room temperature for 24 h. The reaction mixture was

poured onto ice and treated with HCl 2N and $NaHCO_3$ 5%. The organic layers were washed to neutrality with brine, dried over anhydrous Na_2SO_4, filtered and evaporated to dryness. The residue was purified by silica preparative chromatography (hexane/EtOAc 2:8) to afford **124** (56 mg, 55%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.21 (3H, *s*), 3.10 (3H, *s*), 3.44 (3H, *s*), 3.85 (3H, *s*), 3.88 (3H, *s*), 5.75 (1H, *s*), 6.96 (2H, *d, J* = 8.8), 7.55 (2H, *d, J* = 8.8), 7.89 (1H, *s*), 8.21 (1H, *s*). ^{13}C NMR (100 MHz, $CDCl_3$): δ 24.8 (CH_3), 39.5 (CH_3), 55.6 (CH_3), 55.8 (CH_3), 56.0 (CH_3), 105.9 (CH), 108.9 (CH), 114.0 (2CH), 122.5 (C), 127.1 (C), 130.4 (C), 130.8 (2CH), 144.8 (C), 148.8 (C), 163.5 (C), 168.6 (C). HRMS ($C_{18}H_{22}N_2O_6S + H^+$): calcd 395.1271 ($M + H^+$), found 395.1280.

***N*-(3,4-dimethoxyphenyl)-4-nitrobenzenesulfonamide (23).** To a solution of 3,4-dimethoxyaniline (2.82 g, 18.41 mmol) in CH_2Cl_2 (50 mL) and pyridine (4 mL), 4-nitrobenzenesulfonyl chloride was slowly added (4.49 g, 20.25 mmol) and stirred at room temperature. After 4 h, the reaction was treated with HCl 2N and $NaHCO_3$ 5%, washed with brine to neutrality, dried over anhydrous Na_2SO_4 and concentrated under vacuum to yield 5.43 g (87%) of the sulfonamide **23**. The residue was crystallized in EtOAc to afford the purified compound (4.40 g, 70%). M.p.: 181-183 °C (EtOAc). IR (KBr): 3251, 1532, 1450, 803 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.83 (3H, *s*), 3.84 (3H, *s*), 6.38 (1H, *s*), 6.43 (1H, *dd, J* = 8.4 and 2.4), 6.69 (1H, *d, J* = 8.4), 6.77 (1H, *d, J* = 2.4), 7.87 (2H, *d, J* = 8.8), 8.28 (2H, *d, J* = 8.8). ^{13}C NMR (100 MHz, acetone-d6): δ 55.1 (CH_3), 55.2 (CH_3), 107.6 (CH), 111.9 (CH), 114.9 (CH), 124.1 (2CH), 128.6 (2CH), 129.5 (C), 145.2 (C), 147.6 (C), 149.6 (C), 150.2 (C). HRMS ($C_{14}H_{14}N_2O_6S + Na^+$): calcd 361.0465 ($M + Na^+$), found 361.0469.

***N*-(4,5-dimethoxy-2-nitrophenyl)-4-nitrobenzenesulfonamide (334).** To a solution of **23** (112 mg, 0.33 mmol) in CH_3CN (50 mL) tertbutyl nitrite (21.9 µL, 0.16 mmol) was added and stirred at 45°C. After 24 h, additional 0.16 mmol of tertbutyl nitrite was added to the reaction mixture and it was stirred at 45°C for another 24 h. Then, the mixture was concentrated, re-dissolved in EtOAc and treated with 5% $NaHCO_3$ solution. The organic layers were washed with brine, dried over Na_2SO_4 and concentrated under vacuum to yield **334** (109 mg, 86%). The residue was purified by crystallization in MeOH to afford 30 mg (24%). M.p.: 169-172 °C (MeOH). ^{1}H NMR (200 MHz, acetone-d6): δ 3.88 (3H, *s*), 3.98 (3H, *s*), 7.28 (1H, *s*), 7.57 (1H, *s*), 8.16 (2H, *d, J* = 9), 8.41 (2H, *d, J* = 9). ^{13}C NMR (100 MHz, Acetone-d6): δ 55.7 (CH_3), 56.1 (CH_3), 105.3 (CH),

107.7 (CH), 124.6 (2CH), 127.4 (C), 128.9 (2CH), 144.4 (C), 146.5 (C), 150.7 (C), 150.7 (C), 155.3 (C). HRMS ($C_{14}H_{13}N_3O_8S + Na^+$): calcd 406.0316 ($M + Na^+$), found 406.0313.

4-amino-*N*-(3,4-dimethoxyphenyl)benzenesulfonamide (26). To a solution of **23** (1.96 g, 5.81 mmol) in ethyl acetate (150 mL) an MeOH (5 mL), Pd (C) (10 mg) was added and the reaction was stirred at room temperature under H_2 atmosphere for 48 h. By filtration through celite and solvent evaporation, 1.71 g (95%) of **26** was obtained and purified by crystallization in EtOAc (1.39 g, 78%). M.p.: 186-187 °C (EtOAc). IR (KBr): 3449, 3221, 1462, 804 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 3.73 (3H, *s*), 3.76 (3H, *s*), 4.01 (2H, *s*), 6.02 (1H, *s*), 6.41 (1H, *dd, J* = 8.4 and 2.4), 6.52 (2H, *d, J* = 8.4), 6.61 (1H, *d, J* = 8.4), 6.62 (1H, *d, J* = 2.4), 7.40 (2H, *d, J* = 8.4). ^{13}C NMR (100 MHz, acetone-d6): δ 55.0 (CH_3), 55.3 (CH_3), 106.9 (CH), 111.9 (CH), 112.8 (2CH), 113.8 (CH), 126.0 (C), 129.1 (2CH), 131.5 (C), 146.7 (C), 149.4 (C), 152.5 (C). HRMS ($C_{14}H_{16}N_2O_4S + Na^+$): calcd 331.0723 ($M + Na^+$), found 331.0733.

***N*-(3,4-dimethoxyphenyl)-4-(dimethylamino)benzenesulfonamide (29).** To a solution of p-formaldehyde (1.25 g, 41.11 mmol) in MeOH (40 mL) few drops of acetic acid were added to acid pH, then, 1.27 g of **26** (4.11 mmol) were added and stirred for 30 min. Finally, $NaCNBH_3$ (517 mg, 24.66 mmol) was added and the reaction was heated at reflux for 24 h. The reaction mixture was concentrated, re-dissolved in EtOAc and treated with HCl 2N and 5% $NaHCO_3$ solutions. The organic layers were washed with brine to neutrality, dried over Na_2SO_4, filtered through celite and the solvent evaporated in vacuum to afford 1.04 g (75%) of **29**. M.p.: 161-163 °C (EtOAc). IR (KBr): 3467, 3255, 1596, 795 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 3.01 (6H, *s*), 3.79 (3H, *s*), 3.82 (3H, *s*), 6.09 (1H, *s*), 6.48 (1H, *dd, J* = 8.4 and 2.4), 6.57 (2H, *d, J* = 9.2), 6.68 (1H, *d, J* = 8.4), 6.70 (1H, *d, J* = 2.4), 7.51 (2H, *d, J* = 9.2). ^{13}C NMR (100 MHz, $CDCl_3$): δ 39.9 ($2CH_3$), 55.8 (CH_3), 55.9 (CH_3), 107.2 (CH), 110.6 (2CH), 111.1 (CH), 114.9 (CH), 123.7 (C), 129.1 (2CH), 130.2 (C), 146.7 (C), 148.9 (C), 152.8 (C). HRMS ($C_{16}H_{20}N_2O_4S + H^+$): calcd 337.1217 ($M + H^+$), found 337.1204.

***N*-(4,5-dimethoxy-2-nitrophenyl)-4-(dimethylamino)benzenesulfonamide (33).** To sulfonamide **29** (1.17 g, 3.48 mmol) in CH_3CN (50 mL) tertbutyl nitrite was added dropwise by two successive addition (460 µL, 3.48 mmol) and the reaction stirred for 24 h at 45°C. Then, the mixture was concentrated, re-dissolved

in EtOAc and treated with 5% $NaHCO_3$ solution. The organic layers were washed with brine, dried over Na_2SO_4 and concentrated under vacuum to produce 1.22 g of crude reaction product from which 949 mg of **33** (71%) was isolated by crystallization in EtOAc. M.p.: 190-195 °C (EtOAc). IR (KBr): 3255, 1593, 1525, 793 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 3.01 (6H, *s*), 3.86 (3H, *s*), 3.97 (3H, *s*), 6.57 (2H, *d, J* = 9.2), 7.35 (1H, *s*), 7.54 (1H, *s*), 7.63 (2H, *d, J* = 9.2). ^{13}C NMR (100 MHz, $CDCl_3$): δ 40.0 ($2CH_3$), 56.2 (CH_3), 56.7 (CH_3), 102.4 (CH), 107.1 (CH), 110.8 (2CH), 122.9 (C), 129.1 (C), 129.2 (2CH), 131.2 (C), 144.7 (C), 153.2 (C), 155.4 (C). HRMS ($C_{16}H_{19}N_3O_6S + H^+$): calcd 382.1067 ($M + H^+$), found 382.1067.

***N*-(2-amino-4,5-dimethoxyphenyl)-4-(dimethylamino)benzenesulfonamide (35).** To nitroderivative **33** (655 mg, 1.72 mmol) in ethyl acetate (100 mL) under H_2 atmosphere, Pd (C) (10 mg) was added and the reaction stirred at room temperature for 48 h. After filtration through celite and solvent evaporation 577 mg of **35** (95%) was isolated. M.p.: 153-157 °C (MeOH). IR (KBr): 3403, 1449, 824 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 3.02 (6H, *s*), 3.49 (3H, *s*), 3.80 (3H, *s*), 5.77 (1H, *s*), 6.00 (1H, *s*), 6.29 (1H, *s*), 6.61 (2H, *d, J* = 9.2), 7.54 (2H, *d, J* = 9.2). ^{13}C NMR (100 MHz, $CDCl_3$): δ 41.2 ($2CH_3$), 56.8 (CH_3), 57.3 (CH_3), 102.1 (CH), 110.0 (CH), 111.8 (2CH), 114.3 (C), 124.9 (C), 130.5 (2CH), 139.9 (C), 142.5 (C), 150.5 8C), 154.1 (C). HRMS ($C_{16}H_{21}N_3O_4S + H^+$): calcd 352.1326 ($M + H^+$), found 352.1320.

***N*-(2-amino-4,5-dimethoxyphenyl)-4-(dimethylamino)-*N*-methylbenzenesulfonamide (118).** To a solution of **35** (155 mg, 0.44 mmol) in CH_3CN (25 mL) 61 mg of crushed KOH (0.88 mmol) and 55 µL of methyl iodide (0.88 mmol) were added and stirred at room temperature for 24 h. Then, the reaction mixture was concentrated, re-dissolved in CH_2Cl_2, washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated in vacuum to produce 144 mg of crude reaction product from which 105 mg of **118** (65%) was isolated by flash chromatography (hexane/EtOAc 4:6). M.p.: 156-157 °C (MeOH). IR (KBr): 3437, 1462, 812 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 3.04 (6H, *s*), 3.08 (3H, *s*), 3.47 (3H, *s*), 3.82 (3H, *s*), 5.87 (1H, *s*), 6.33 (1H, *s*), 6.65 (2H, *d, J* = 9.2), 7.52 (2H, *d, J* = 9.2). ^{13}C NMR (100 MHz, $CDCl_3$): δ 38.8 (CH_3), 39.9 ($2CH_3$), 55.7 (CH_3), 56.3 (CH_3), 100.5 (CH), 110.5 (2CH), 111.0 (CH), 118.7 (C), 121.8 (C), 129.9 (2CH), 140.3 (C), 140.8 (C), 149.8 (C), 152.9 (C). HRMS ($C_{17}H_{23}N_3O_4S + H^+$): calcd 366.1482 ($M + H^+$), found 366.1471.

***N*-(2-((4-(dimethylamino)-*N*-methylphenyl)sulfonamido)-4,5-dimethoxyphenyl) formamide (132).** The compound **118** (60 mg, 0.16 mmol) was stirred in a mixture of CH_2Cl_2 (30 mL), pyridine (1 mL) and formic acid (2 mL) at room temperature for 24 h. Then, the reaction mixture was poured onto ice and treated with HCl 2N and $NaHCO_3$ 5%, washed to neutrality with brine, dried over anhydrous Na_2SO_4, filtered and solvent evaporated to produce 51 mg of crude reaction product. The residue was purified by silica preparative chromatography (hexane/EtOAc 2:8) to afford **132** (31 mg, 50%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.05 (6H, *s*), 3.09 (3H, *s*), 3.48 (3H, *s*), 3.91 (3H, *s*), 5.85 (1H, *s*), 6.65 (2H, *d, J* = 9.2), 7.45 (2H, *d, J* = 9.2), 8.00 (1H, *s*), 8.42 (1H, *s*). ^{13}C NMR (100 MHz, $CDCl_3$): δ 39.4 (CH_3), 40.1 ($2CH_3$), 55.8 (CH_3), 56.0 (CH_3), 105.5 (CH), 109.1 (CH), 110.5 (2CH), 120.2 (C), 123.0 (C), 129.9 (2CH), 130.3 (C), 144.9 (C), 148.6 (C), 153.2 (C), 159.1 (CH). HRMS ($C_{18}H_{23}N_3O_5S + Na^+$): calcd 416.1251 ($M + Na^+$), found 416.1250.

4-((5,6-dimethoxy-1H-benzo[d][1,2,3]triazol-1-yl)sulfonyl)-*N*,*N*-dimethylaniline (117). To a solution of **35** (100 mg, 0.28 mmol) in MeOH (20 mL) and H_2O (200 µL) at 0°C, tertbutyl nitrite (33.8 µL, 0.28 mmol) was added and the reaction stirred. After 1 h, acetic acid (20 µL) was added to the reaction mixture and stirred for 24 h to room temperature. Then, the mixture was concentrated, re-dissolved in EtOAc and treated with 5% $NaHCO_3$ solution. The organic layers were washed with brine to neutrality, dried over Na_2SO_4 and concentrated under vacuum. The residue was chromatographed on silica preparative (hexane/EtOAc 1:1) to afford the purified compound **117** (14 mg, 13%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.03 (6H, *s*), 3.94 (3H, *s*), 4.04 (3H, *s*), 6.62 (2H, *d, J* = 9.2), 7.34 (1H, *s*), 7.46 (1H, *s*), 7.88 (2H, *d, J* = 9.2). ^{13}C NMR (100 MHz, $CDCl_3$): δ 40.0 ($2CH_3$), 56.2 (CH_3), 56.6 (CH_3), 92.7 (CH), 99.1 (CH), 110.9 (2CH), 120.8 (C), 127.1 (C), 130.0 (2CH), 139.6 (C), 149.2 (C), 152.7 (C), 154.2 (C). HRMS ($C_{16}H_{18}N_4O_4S + H^+$): calcd 363.1122 ($M + H^+$), found 363.1127.

Methyl 3,5-dinitrobenzoate (80). 3.24 g of 3,5-dinitrobenzoic acid (15.28 mmol) was stirred in a mixture of MeOH (100 mL) and H_2SO_4 (1 mL) for 12 h. Then, anhydrous Na_2CO_3 was added to the reaction mixture and concentrated in vacuum. The residue was re-dissolved in EtOAc, washed with water to neutrality, dried over Na_2SO_4 and solvent evaporated. 3.22 g (94%) of the crude reaction product was obtained and used

without further purification. ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.06 (3H, *s*), 9.18 (2H, *d, J* = 2.4), 9.24 (1H, *t, J* = 2.4). GC-MS ($C_8H_6N_2O_6$): calcd 226, found 226.

Methyl 3,5-diaminobenzoate (81). The compound **80** (3.23 g, 14.27 mmol) was suspended in ethyl acetate (100 mL) and was palladium-catalyzed (Pd (C) 10 mg) reduced under H_2 atmosphere for 72 h. The reaction mixture was filtered through Celite© and the solvent evaporated in vacuum to isolate 2.18 g (92%) of **81**. Crude reaction product was obtained and used without further purification. ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.85 (3H, *s*), 6.18 (1H, *t, J* = 2), 6.77 (2H, *d, J* = 2). GC-MS ($C_8H_{10}N_2O_2$): calcd 166, found 166.

Methyl 3-amino-5-((4-methoxyphenyl)sulfonamido)benzoate (84A) and methyl 3,5-bis((4-methoxyphenyl)sulfonamido)benzoate (84B). To a solution of **81** (1.52 g, 9.14 mmol) in CH_2Cl_2 (50 mL) and pyridine (1 mL), was dropwise added 4-methoxybenzenesulfonyl chloride (1.89 g, 9.14 mmol) dissolved in CH_2Cl_2 (20 mL). The mixture was stirred at room temperature for 4 h. Then the reaction was treated with HCl 0.5N washed with brine, dried over anhydrous Na_2SO_4 and concentrated in vacuum. The residue was purified by flash chromatography on silica gel with toluene/EtOAc (7:3) to yield compounds **84A** (375 mg, 12%) and **84B** (957 mg, 41%). **84A:** M.p.: 165-166 °C (CH_2Cl_2/Hexane). IR (KBr): 3468, 3377, 1697, 1497, 1176, 803 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.80 (3H, *s*), 3.84 (3H, *s*), 6.82 (1H, *t, J* = 2), 6.87 (2H, *d, J* = 8.8), 7.01 (1H, *t, J* = 2), 7.09 (1H, *t, J* = 2), 7.71 (2H, *d, J* = 8.8). ^{13}C NMR (100 MHz, CD_3OD): δ 51.1 (CH_3), 54.7 (CH_3), 110.2 (CH), 110.8 (CH), 111.5 (CH), 113.8 (2CH), 128.9 (2CH), 130.9 (C), 131.2 (C), 138.8 (C), 148.9 (C), 163.1 (C), 167.0 (C). HRMS ($C_{15}H_{16}N_2O_5S + H^+$): calcd 337.0853 ($M + H^+$), found 337.0855. **84B:** M.p.: 174-178 °C (CH_2Cl_2/Hexane). IR (KBr): 3270, 1724, 1498, 1150, 802 cm^{-1}. ^{1}H NMR (400 MHz, CD_3OD): δ 3.81 (9H, *s*), 6.94 (4H, *d, J* = 8.8), 7.31 (2H, *d, J* = 2), 7.35 (1H, *t, J* = 2), 7.63 (4H, *d, J* = 8.8). ^{13}C NMR (100 MHz, CD_3OD): δ 51.5 (CH_3), 54.8 ($2CH_3$), 113.8 (4CH), 115.1 (CH), 116.0 (2CH), 129.0 (4CH), 130.6 (2C), 131.5 (C), 139.1 (2C), 163.2 (2C), 165.9 (C). HRMS ($C_{22}H_{22}N_2O_8S_2 + Na^+$): calcd 529.0710 ($M + Na^+$), found 529.0749.

Methyl 3,5-bis((4-methoxy-*N*-methylphenyl)sulfonamido)benzoate (147). To a solution of **84B** (132 mg, 0.39 mmol) in acetone (20 mL) K_2CO_3 (542 mg, 3.92 mmol) and $(CH_3)_2SO_4$ (281 µL, 2.94 mmol) were added, heated at reflux and stirred overnight. Then, the reaction mixture was filtered, poured onto ice and

extracted with CH_2Cl_2. The organic layers were dried over anhydrous Na_2SO_4, filtered and evaporated to dryness. By crystallization in MeOH compound **147** (73 mg, 51%) was isolated. M.p.: 139-142 °C (MeOH). ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.10 (6H, *s*), 3.84 (6H, *s*), 3.86 (3H, *s*), 6.91 (4H, *d, J* = 8.8), 7.21 (1H, *t, J* = 2), 7.45 (4H, *d, J* = 8.8), 7.66 (2H, *d, J* = 2). ^{13}C NMR (100 MHz, $CDCl_3$): δ 38.1 ($2CH_3$), 52.9 (CH_3), 56.1 ($2CH_3$), 114.7 (4CH), 126.0 (2CH), 127.9 (2C), 129.4 (CH), 130.3 (4CH), 131.7 (C), 142.9 (2C), 163.7 (2C), 165.9 (C). HRMS ($C_{24}H_{26}N_2O_8S_2 + Na^+$): calcd 557.1023 ($M + Na^+$), found 557.1024.

Methyl 3-amino-5-methoxybenzoate (78). 957 mg of 3-amino-5-methoxybenzoic acid (5.73 mmol) was stirred in a mixture of MeOH (50 mL) and H_2SO_4 (1 mL) for 24 h. Then, anhydrous Na_2CO_3 was added to the reaction mixture, filtered and concentrated in vacuum. The residue was re-dissolved in EtOAc, filtered again and evaporated to dryness. 799 mg (77%) of crude reaction product was obtained and used without further purification. ^{1}H NMR (400 MHz, CD_3OD): δ 3.74 (3H, *s*), 3.83 (3H, *s*), 6.48 (1H, *t, J* = 2), 6.84 (1H, *t, J* = 2), 6.95 (1H, *t, J* = 2). ^{13}C NMR (100 MHz, $CDCl_3$): δ 52.1 (CH_3), 55.3 (CH_3), 104.3 (CH), 105.7 (CH), 109.2 (CH), 132.0 (C), 147.6 (C), 160.6 (C), 167.1 (C). GC-MS ($C_9H_{11}NO_3$): calcd 181, found 181.

Methyl 3-methoxy-5-((4-methoxyphenyl)sulfonamido)benzoate (79). To a solution of **78** (620 mg, 3.42 mmol) in CH_2Cl_2 (50 mL) and pyridine (2 mL), was slowly added 4-methoxybenzenesulfonyl chloride (707 mg, 3.42 mmol). The mixture was stirred at room temperature for 4 h. Then the reaction was treated with HCl 2N and $NaHCO_3$ 5%, washed with brine, dried over anhydrous Na_2SO_4 and the solvent evaporated to obtain 1.15 g (95%) of the sulfonamide **79**. The crude reaction product was purified by crystallization in CH_2Cl_2 (529 mg, 44%). M.p.: 176-177 °C (CH_2Cl_2). IR (KBr): 3258, 1700, 1497, 1152, 802 cm^{-1}. ^{1}H NMR (400 MHz, CD_3OD): δ 3.76 (3H, *s*), 3.81 (3H, *s*), 3.85 (3H, *s*), 6.91 (1H, *t, J* = 2.4), 6.99 (2H, *d, J* = 8.8), 7.20 (1H, *t, J* = 2.4), 7.32 (1H, *t, J* = 2.4), 7.71 (2H, *d, J* = 8.8). ^{13}C NMR (100 MHz, acetone-d6): δ 51.6 (CH_3), 54.9 (CH_3), 55.1 (CH_3), 109.6 (CH),110.2 (CH), 113.1 (CH), 114.2 (2CH), 129.2 (2CH), 131.2 (C), 132.1 (C), 139.6 (C), 160.3 (C), 163.2 (C), 165.6 (C). HRMS ($C_{16}H_{17}NO_6S + H^+$): calcd 352.0849 ($M + H^+$), found 352.0842.

3-methoxy-5-((4-methoxy-*N*-methylphenyl)sulfonamido)benzoic acid (90). To a solution of **79** (100 mg, 0.28 mmol) in CH_3CN (40 mL) 38 mg of crushed KOH (0.56 mmol) and 36 µL of methyl iodide (0.56

672 mmol) were added and stirred at room temperature for 24 h. Then, the reaction mixture was concentrated,
673 re-dissolved in CH_2Cl_2, treated with HCl 2N, washed with brine to neutrality, dried over anhydrous Na_2SO_4,
674 filtered and concentrated in vacuum. The residue was purified by silica preparative chromatography with
675 CH_2Cl_2 (98:2) yielding compound **90** (69 mg, 66%). M.p.: 186-187 °C (CH_2Cl_2). IR (KBr): 3000, 1689, 1502, 806
676 cm^{-1}. ^1H NMR (400 MHz, CD_3OD): δ 3.16 (3H, *s*), 3.79 (3H, *s*), 3.86 (3H, *s*), 6.94 (1H, *t, J* = 2.4), 7.03 (2H, *d, J* =
677 8.8), 7.26 (1H, *t, J* = 2.4), 7.46 (1H, *t, J* = 2.4), 7.47 (2H, *d, J* = 8.8). ^{13}C NMR (100 MHz, $CDCl_3$): δ 37.8 (CH_3),
678 55.6 (CH_3), 55.7 (CH_3), 113.8 (CH), 114.0 (2CH), 118.7 (CH), 119.3 (CH), 127.7 (C), 129.9 (2CH), 130.9 (C),
679 143.1 (C), 159.7 (C), 163.2 (C), 170.6 (C). HRMS ($C_{16}H_{17}NO_6S + H^+$): calcd 352.0849 ($M + H^+$), found 352.0848.

680 **2.2 Determination of aqueous solubility**

681 The aqueous solubility of the sulfonamides was determined in a Helios Alfa Spectrophotometer using an
682 approach based on the saturation shake-flask method. 1-2 mg of each tested compound was suspended in
683 300 µL pH 7.0 buffer and stirred for 72 h at room temperature. The resulting mixture was filtrated over a 45
684 µm filter to discard the insoluble residues. Then, a scan between 270 and 400 nm was performed and the
685 three maximum wavelengths of absorbance of each compound were selected. Calibration lines were
686 performed at these wavelengths and the concentration in the supernatant was measured by UV
687 absorbance.

688 **2.3 Cells and culture conditions**

689 The *Leishmania* strains used in this study were PER/ES/2013/ATE1FL6 and MCAN/ES/MON1/Z001 of
690 *Leishmania infantum.* The macrophage human cell line used, originally obtained from a patient with
691 histiocytic leukemia was U937 (ATCC® CRL1593.2) and human tumor cell lines were HT-29, HeLa, and MCF7,
692 obtained from a patient with colon, cervical, and breast cancer respectively.

693 *Leishmania* promastigotes were cultured at 27°C in RPMI 1640 supplemented with L-glutamine (Lonza-
694 Cambrex, Karlskoga, Sweden), 10 % heat-inactivated fetal bovine serum (HIFBS) (Lonza-Cambrex), and 100
695 µg/mL streptomycin-100 IU/mL penicillin (Lonza-Cambrex) in 25 mL culture flasks. Logarithmic and late

696 stationary promastigotes were obtained after incubation of the parasites for 3-4 and 6-7 days respectively
697 with a starting inoculum of $4 \cdot 10^6$ parasites/mL.

698 *Leishmania* axenic amastigotes were obtained from late stationary promastigotes. Once promastigotes had
699 been centrifuged at 250 g for 10 min a percoll gradient is made to select the living population. Then,
700 promastigotes were seeded at $4 \cdot 10^6$ parasites/mL in M199 medium (Invitrogen, Leiden, The Netherlands)
701 supplemented with 10% HIFBS, 1 g/L β-alanine, 100 mg/L L-asparagine, 200 mg/L saccharose, 200 mg/L D-
702 fructose, 50 mg/L sodium pyruvate, 320 mg/L malic acid, 40 mg/L fumaric acid, 70 mg/L succinic acid, 200
703 mg/L α-ketoglutaric acid, 300 mg/L citric acid, 1.1 g/L sodium bicarbonate, 5 g/L morpholineethanesulfonic
704 acid (MES), 0.4 mg/L hemin, 10 mg/L gentamicin and 100 µg/mL streptomycin-100 IU/mL penicillin; pH 5.4,
705 at 37°C in humidified 95% air and 5% CO_2 atmosphere. After 24h of incubation, all parasites had a rounded
706 morphology without a flagellum.

707 U937 (human lung histiocytic lymphoma) and HT-29 (human colon carcinoma) cells were cultured at 37°C in
708 complete RPMI 1640 medium (see above) in humidified 95% air and 5% CO_2 atmosphere. HeLa (human
709 cervical carcinoma) and MCF7 (human breast carcinoma) cell lines were cultured in DMEM culture medium
710 containing 10% (v/v) HIFBS, 2 mM L-glutamine and 100 µg/mL streptomycin-100 IU/mL penicillin at 37 °C in
711 humidified 95% air and 5% CO_2.

712 **2.4 Cytotoxicity assays**

713 The effect of the different compounds on the proliferation of human tumor cell lines was determined by
714 using the XTT (sodium 3,39-[1(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene
715 sulfonic acid hydrate) cell proliferation kit (Roche Molecular Biochemicals, Mannheim, Germany) as
716 previously described (Scudiero et al., 1988). Briefly, a freshly prepared mixture solution of XTT labeling
717 reagent and PMS (N-methyl-dibenzopyrazine methyl sulfate) electron coupling reagent was added to cells
718 and were incubated during the corresponding time according to each cell line (6 h for U937 and HT-29 and
719 4 h for HeLa and MCF7 cells), in a humidified atmosphere (37 °C, 5% CO_2), and the absorbance of the
720 formazan product generated was measured at a test wavelength of 450 nm. A positive control is formed by

cells without compounds at 72 h and a negative control is formed by cells without compounds at 0 h of incubation. Measurements were performed in triplicate, and each experiment was repeated three times.

Cell viability was evaluated seeding 100 µL of cells in exponential growth phase with appropriate cell line concentration ($1 \cdot 10^5$ U937 cells/mL, $3 \cdot 10^4$ HT-29 cells/mL, $1.5 \cdot 10^4$ HeLa cells/mL and $1.5 \cdot 10^4$ MCF7 cells/mL) in complete RPMI 1640 or DMEM medium in 96-well plates at 37°C and 5% CO_2. The tested sulfonamides were added after 24 h incubation, to allow cells to attach to the plates, at 10 µM concentration for U937 cell line and 1 µM concentration for HT-29, HeLa, and MCF7 cells lines, and the effect on the proliferation was evaluated 72 h post-treatment. Compounds were dissolved in dimethyl sulfoxide (DMSO) and the final solvent concentrations never exceeded 0.5% (v/v).

2.5 Leishmanicidal assays

The leishmanial growth inhibition assay in promastigotes and axenic amastigotes was performed by using the XTT method described above.

***In vitro* promastigote assay.** *In vitro* promastigote susceptibility assay was performed with logarithmic and late stationary promastigotes in two different assays. 100 µL of promastigotes in complete RPMI 1640 medium were seeded at $4 \cdot 10^6$ parasites/mL in 96-well plates at 27°C, in the absence and the presence of 10 µM concentration of the indicated sulfonamides. The compounds were dissolved in DMSO and the final solvent concentrations never exceeded 0.5% (v/v). After 24 h incubation, each plate-well was then examined by light microscopy for detecting changes in parasite morphology or motility. 72 h after treatment, 50 µL of XTT solution were added to each well, cells were incubated for 7 h at 27°C and thereafter, absorbance was measured at 450 nm with a microplate spectrophotometer. Measurements were done in triplicate, and each experiment was repeated three times.

Axenic amastigote assay. Axenic amastigote viability assays were performed following a similar method. Promastigotes were differentiated into axenic amastigotes as previously described. 100 µL of late stationary promastigotes were seeded at $4 \cdot 10^6$ parasites/mL in complete M199 medium in 96-well plates at

37°C. After 24 h incubation sulfonamides at different concentrations (a first screening using 10 µM and then 15, 10, 9, 7, 6, 4, 2, and 1 µM concentration for active compounds) were added over the axenic amastigotes. Then, the XTT solution was added 48 h post-treatment and incubated for 7 h at 37°C. The efficacy of each compound was estimated by calculating the IC_{50} (half maximal inhibitory concentration). Measurements were done in triplicate, and each experiment was repeated three times.

***In vitro* macrophage infection and intracellular amastigote assay.** The in vitro infection of the human U937 myeloid cell line with *L. infantum* promastigotes was carried out to evaluate the antileishmanial activity of new sulfonamides. U937 cells were centrifuged at 250 g for 10 min. Then, 400 µL of cells in complete RPMI 1640 medium were seeded at $3.75 \cdot 10^5$ cells/mL in 8-well chambers slides (LabTek, New York, NY) at 37°C and were differentiated by stimulation with 20 ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma, Saint Louis, MO) for 72 h. After this time, monocytes had been differentiated into macrophages and therefore, cells had adhered to the surface of the well. The cultures were rinsed three times with complete RPMI medium to remove nondifferentiated cells. Then, infection was performed by incubation with late stationary *L. infantum* promastigotes at 37°C at a 5:1 promastigote:macrophage ratio in 400 µL complete RPMI medium in an atmosphere of 5% CO_2 for 2 h. Noninternalized promastigotes were removed by 3-4 successive washes with complete RPMI medium. Then, infected macrophages were incubated in complete culture medium with sulfonamides at different concentrations (a first screening using 20 µM and then 20, 15, 10, 7, and 5 µM concentration for active compounds) for 48 h. The compounds were dissolved in DMSO and the final solvent concentrations never exceeded 0.5% (v/v). Finally, fixation and staining were performed. The wells were washed three times with fresh complete medium, then, the cells were treated with hypotonic solution (complete medium:water 9:11) for 5 min and were fixed with 150 µL ethanol:acetic acid 3:1 for 10 min (step repeated three times). The preparations were allowed to air dry and the wells were removed from the slide. Modified Giemsa staining was carried out with Diff-Quick® Stain Solution I and II (Dade Behring, Marburg, Germany). The preparations were washed with distilled water, air-dried, and mounted with Entellan® Neu (Merck, Darmstadt, Germany). The number of amastigotes per infected cell was estimated by counting 100 cells per biological replicate

randomly. The experiment was performed in triplicate and the statistical analysis was based on Student's paired t-test.

2.6 Docking studies

The sequences of α and β tubulins from *Leishmania* with sizes larger than 400 amino acids were retrieved from UniProt (Bateman, 2019). Sequences were aligned with each other and with the tubulin sequence of sheep tubulin sequence used in the X-ray structure from the Protein Data Bank (Berman et al., 2003) with pdbID 3HKC using ClustalX (Larkin et al., 2007). The amino acids forming the colchicine domain were selected as those closer to 6 Å to the ligands in the colchicine site of the X-ray structures of tubulin in complex with colchicine site ligands published in the pdb. The comparison of 20 Leishmania sequences with the sheep sequence indicated amino acid changes at 11 residues with sidechains in contact with colchicine site ligands. Eight of them were conserved in the Leishmania sequences and the three that showed differences (i.e. N167β, K254β, and I347β) could be represented by just four sequence combinations that were used in the building of the homology models. The sheep 3HKC X-ray structure was used as a template for the generation of the homology models as the ABT-751 ligand is one of the very few ligands binding to the 3 zones (1-3) of the colchicine site and has an *N*-aryl-methoxybenzenesulfonamide structure in common to the compounds in our library, thus providing the most favorable starting point for the docking studies, as confirmed by successful cross-dockings of other ligands with known X-ray structures binding at zones 1, 2, or 3, such as combretastatin A-4 (sites 1 and 2) or nocodazole (sites 2 and 3). 5 homology models were generated with Modeller 9.15 (Šali and Blundell, 1993) for each sequence combination, for a total of 20 homology models for the Leishmania proteins. The models were manually curated to avoid the collapse of the colchicine binding site previous to the docking experiments. Docking studies of the ligands in the mammalian proteins and the *Leishmania* homology models were carried out as previously described (Álvarez et al., 2013). Additionally, representative ligands binding at the 1, 2, and/or 3 zones of the colchicine site were docked and compared with their X-ray complex structures to validate the selection of 3HKC as a template. Docking runs were performed with PLANTS with default settings (Korb et al., 2009) and generated 10 runs per ligand. AutoDock 4.2 (Forli et al., 2016) runs applied the Lamarckian genetic

algorithm (LGA) 100−300 times for a maximum of 2.5×10^6 energy evaluations, 150 individuals, and 27000 generations maximum. The poses were automatically assigned to zones 1-3, and the results tabulated using in-house KNIME pipelines (Berthold et al., 2007). Z-scores were generated from the programs' scores. The results were analyzed with Chimera (Pettersen et al., 2004), Marvin ("Marvin 17.8 ChemAxon," 2017), OpenEye ("OpenEye Scientific Software, Inc, Santa Fe," 2019), and with JADOPPT (Garcia-Perez et al., 2017).

3. Results and discussion

3.1 Chemical library design

The search for new antiparasitic drugs, including antileishmanial drugs as well, has followed two distinct approaches, the blind screening of large compound libraries (HTS) that impair the viability of the parasite, the so-called phenotypic assays, or target based screenings where ligands acting on a particular target of interest are sought (Zulfiqar et al., 2017). The first approach has the advantage of guaranteeing effects on the whole organism and therefore fulfills pharmacokinetic and pharmacodynamic requirements and finds drugs active against unforeseen targets, but has the disadvantage of needing an often difficult deconvolution of the target in the following drug development process. Target-based screens, on the other hand, have the advantage of facilitating later stages of drug development, but often the compounds do not have adequate pharmacokinetic properties, or the target turns out to be non-essential and lacks activity in the whole organism. Here, we have adopted an intermediate approach designing a focused library against a well validated target, tubulin, and assaying the effects of the compounds in a phenotypic screen against several stages of the parasite life cycle. As a result, both pharmacokinetic and pharmacodynamic issues are simultaneously assessed and a sound basis of the mechanism of action is achieved.

Trypanosomatid microtubules have been shown to present up to 11 different amino acid substitutions in the colchicine-binding site compared to mammalian orthologs (Luis et al., 2013). To determine sequence differences from humans to *Leishmania* potentially affecting the binding of colchicine site ligands, the colchicine site was defined as formed by any tubulin residue whose sidechain is closer than

6 Å to any of the more than 50 colchicine site ligands in complex with tubulin published in the Protein Data Bank (Berman et al., 2003; Vicente-Blázquez et al., 2019). Clustal X (Larkin et al., 2007) alignment of the X-ray structure sequences with the Leishmania sequences retrieved from UniProt (Bateman, 2019) and comparison of the amino acids assigned to the colchicine site identified mutated residues (Fig 1). The colchicine domain in tubulin has been subdivided into three sub-pockets, called zones 1-3, that bind distinct moieties of typical colchicine site ligands, such as combretastatin A-4 (Fig 1) and nocodazole. Zone 1 (Massarotti et al., 2012) is the pocket for combretastatin A-4 ring B. There is high sequence conservation between Leishmanial and human tubulin in this pocket, with the sole exception of the A316βS replacement, located at the inner edge of the V-shaped A and B rings of combretastatin A-4 and which makes the gap smaller but more polar due to the presence of the serine side chain hydroxyl group. Therefore, small modifications were envisaged for B-rings, except for the introduction of non-bulky hydrogen bond acceptors and donors such as small amine or formamide groups. *Leishmania* tubulin sequences show an A250βS change compared to the cow ortholog in the pocket accommodating the bridge connecting zones 1 and 2, located in a flexible loop at the interface between tubulin subunits. The presence of the hydroxyl group in this region suggested the introduction of a sulfonamide to bridge the A and B rings. *Leishmania* tubulin sequences contain the substitutions A316βS, A354βS, C241βT, and V318βL in zone 2. These changes make for a smaller and more polar pocket when compared to the mammalian protein due to the presence of three additional hydroxyl groups. This suggested the possibility of removing some of the methoxyl groups from the classical trimetoxyphenyl A ring of combretastatin A-4 and the introduction of additional hydrogen bonding groups. These modifications may also probably reduce activity against human tubulin, thus conferring selectivity. Finally, the N167βI and the Y202βM substitutions were observed in zone 3 which would become smaller and less polar, thus leaving the negatively charged D200β in a very hydrophobic environment. However, mutations in zone 3 have been described in parasites resistant against benzimidazoles, and therefore this zone was not pursued (Furtado et al., 2016).

Considering these sequence differences between the tubulin of Leishmania and the mammalian hosts at the colchicine site, a focused library of 350 compounds was designed by a combination of diverse substitutions on a diarylsulfonamide scaffold and later synthesized. The substitutions were selected to fit

849 the structural requirements of the parasite tubulins and to prevent binding to the mammalian counterparts
850 and are schematically shown in Fig 2, altogether with the general synthetic route applied to build the
851 library. According to the observed sequence differences for zone 2, the trimethoxyphenyl ring of
852 mammalian anti-tubulin ligands has been replaced with dimethoxyphenyl rings with the methoxy groups in
853 different positions, methoxypyridines, carboxyanilines, and others. All the compounds were isolated,
854 purified, and chemically characterized. Here, the synthetic details described in the Methods section and
855 below for the active compounds are described. The synthesized compounds are readily available,
856 chemically stable, and possess drug-like properties (Supp Mat Table 1) (Daina et al., 2017), in good
857 agreement with the requests for new antileishmanial therapies.

858 **3.2 Chemical synthesis**

859 The construction of the diarylsulfonamide scaffolds was performed through the reaction between
860 sulfonyl chlorides and primary amines (Fig 2). The reactions occurred in good yields (85-98%) and could be
861 easily prepared in large amounts for later modifications. Non-commercial amines required for the synthesis
862 of the sulfonamide bridge were obtained by the nitration of the correspondent aromatic rings, and
863 subsequent palladium-catalyzed reduction. When further substituent modifications on the ring were
864 required, they were carried out before the introduction of the amine groups. Occasionally, for aryls with
865 two amino groups or with an amino group and a carboxylic acid group simultaneously, products with three
866 aromatic rings were also obtained (e.g. **63B, 147**). Diarylsullfonamides with amine or amine derivative
867 substituents were prepared after sulfonamide assembly from nitro groups and later functional group
868 modification. Substitutions on the sulfonamide nitrogen were introduced by alkylation reactions.
869 Bromination reactions were performed when necessary. Detailed chemical synthesis of compounds that
870 showed antileishmanial activity can be found in materials and methods and the structures of all the
871 synthesized compounds are available from the authors upon request.

872 **3.3 Solubility and chemical stability**

873 The low stability and low aqueous solubility of 1 µg/mL (Vandermeulen et al., 2006) are the main
874 pharmacokinetic drawbacks of Amphotericin B, which is generally administered in sodium deoxycholate or

liposomal forms and also needs special storing and transport conditions, greatly increasing the cost and difficulty of treatment. All the prepared compounds remained stable for more than 48h in solution at room temperature, as determined by ^{1}H-NMR. Most of them are crystalline solids that remain unaltered for weeks at room temperature. The solubility of representative active compounds of the series in phosphate buffer at pH 7.0 was determined by shaking in pH 7 phosphate buffer for 72h, microfiltration, and quantification by UV absorbance measurements at three different wavelengths to ascertain identity. Compounds **26**, **35**, and **63B** showed thermodynamic solubilities of 55 µg/mL, 118 µg/mL; and, 1889 µg/mL, respectively. These are moderate to good aqueous solubilities, which is an important parameter for the oral administration of drugs.

3.4 Cytotoxicity in human cells

The effect of the synthesized sulfonamides on the cellular proliferation of four human tumor cell lines: U937 (human lung histiocytic lymphoma), HT-29 (human colon carcinoma), HeLa (human cervical carcinoma), and MCF7 (human breast adenocarcinoma), was studied as a surrogate of human toxicity and to select the least cytotoxic compounds against human cells for the *in vitro* leishmanicidal assays (Table 1). Compounds were tested at concentrations of 10 µM for the U937 cell line and 1 µM for HT-29, HeLa, and MCF7 cell lines. Most of the compounds did not show antiproliferative effects at the concentrations studied, as a result of the structural modifications introduced to reduce binding to mammalian tubulin. The more cytotoxic compounds were those with bulky substituents on the sulfonamide nitrogen, such as a benzyl group, in the 3,5-dimethoxyaniline series (e.g. compounds **332, 326B,** and **275**) and/or a bromine atom on the 2,5-dimethoxyaniline series (e.g. **204, 332** and **326B**) (Table 1, Fig 3). After the evaluation of antileishmanial activity (see beow), the IC_{50} values of antiproliferative activity against the human cancer cell line HeLa were determined, and selectivity indexes calculated (Supplemental Table S1). The best selectivity indexes were for compound **276B**, with values of 11.5 for axenic amastigotes and 3.45 for intracellular amastigotes.

3.5 Activity in Leishmania promastigotes

The 350 sulfonamides under study were tested for their antiprotozoal activity against logarithmic and late stationary cultured *L. infantum* promastigotes (MCAN/ES/MON1/Z001). Cell proliferation was quantified 72 h after drug treatment at 10 µM concentration using the XTT method and compared with untreated cells taken as 100% of proliferation. None of the compounds was active against this parasite stage at the tested concentration. However, promastigotes are not the mammalian stage and noticeable differences in gene expression profiles have been found with the intracellular amastigote stage in the mammalian host (Almeida et al., 2004; Leifso et al., 2007).

3.6 Activity in axenic amastigotes

Since the amastigote form of the *Leishmania spp.* is responsible for all clinical manifestations in humans and due to the simplicity of the assay compared to the *in vitro* macrophage infection and intracellular amastigote assay, we first tested our compounds against axenic amastigotes to select the best candidates for the intracellular assay. *L. infantum* axenic amastigotes were differentiated from late stationary promastigotes by temperature, pH, and culture medium variation. All the compounds were tested initially at 10 µM concentration after 48 h of treatment using the XTT method. Those that presented significant activity were selected for the calculation of the IC_{50} values. Measurements were done in triplicate, and each experiment was repeated three times. Eight compounds out of 350 (2.3%) showed potencies better than 10 µM against axenic amastigotes (Table 1), and 3 of them (**129, 204,** and **332**) were equal to or better than miltefosine. Compound **129** was the most potent of the series with sub-micromolar potency and did not show cytotoxicity against the human cancer cell lines. Five additional compounds had satisfactory IC_{50} values between 5-12 µM (**326B, 275, 279, 334** and **276B**) (Table 1). Series with several active compounds are phenyl sulfonamides of 2,5-dimethoxy- (**129, 204** and **332**) and 3,5-dimethoxy- (**275**, **326B**) anilines, the first one including the three most potent compounds. The introduction of a bromo substituent in this series renders the compounds (**204**, **326B,** and **332**) cytotoxic, but fortunately, the most potent compound of the series **129** is not cytotoxic at the tested concentrations. The allowed substituents on the phenylsulfonamide include the methoxy groups and both monomethyl and dimethylamines, with additional substituents on the ring also being tolerated. These results indicate a more permissive binding

926 pocket on the target for these moieties. Interestingly, the larger triaryl compound **276B** is also active
927 against axenic amastigotes and not cytotoxic. These results show that small structural changes can result in
928 selective activity against axenic amastigotes without affecting the host cells. These non-cytotoxic
929 compounds are considered ideal candidates for further development as leishmanicidal drugs.

930 **3.7 Activity against intracellular amastigotes**

931 Intracellular amastigotes are the clinically relevant infective stage of *Leishmania spp*. in mammals
932 and the frequently show different drug sensitivities than promastigotes or axenic amastigotes (Zulfiqar et
933 al., 2017). The intracellular leishmanicidal activity was assayed in amastigote-infected U937 cells. Infection
934 was performed by incubation with late stationary *L. infantum* promastigotes. Then, infected macrophages
935 were incubated with treatments for 48 h. After fixation and staining of the samples, the activity was
936 determined by counting the number of amastigotes per infected cell in a random population of cells. The
937 activity against intracellular amastigotes at 20 µM was evaluated for all the compounds in the library not
938 showing cytotoxicity against the U937 macrophage cell line used for infection at a concentration of 10 µM
939 (157 compounds). 16 compounds showed activity against intracellular amastigotes (Table 1), 10 % of the
940 tested compounds and 5% of the total: very high success rates. More active compounds were found against
941 intracellular amastigotes than axenic amastigotes. Different sensitivities between parasite stages have been
942 previously observed for other families of compounds, but these relative numbers are opposite to the usual
943 findings of promastigotes being more sensitive than amastigotes (De Muylder et al., 2011).

944 Among the 8 active compounds against axenic amastigotes (Table 1), 4 were cytotoxic against U937
945 cells, 2 were inactive against the intracellular amastigotes (i.e. **279** and **334**), and only 2, i.e. **129** and **276B**
946 (Fig 4), were active against both amastigote forms. Box Plot data analysis of the number of amastigotes per
947 cell (Fig 4A) showed that the infection progress is inversely proportional to the ligand concentration when
948 compared with untreated control cells. 75% of the analyzed cells host 0-1 amastigote per cell when treated
949 with active compounds (Fig 4B), whereas the same percentage of untreated cells accommodate 0-4
950 amastigotes per cell. The maximum rate reached 6-9 amastigotes per cell under the different treatments
951 and 15 amastigotes per cell in the untreated control. Hence, late stationary *L. infantum* promastigotes

infected the U937 macrophages and subsequently differentiated into intracellular amastigotes, divided into the host cell, and infected other cells in the untreated control, whereas evidence shown in Table 1 and Figures 4 and 5 suggests that at least one of these steps may have been partially blocked in treated cells by a few compounds. Compound **129** leads to the most remarkable decrease in the infection measured in terms of the percentage of infected cells and the number of amastigotes per infected cell. Sulfonamides **279** and **334** with activity against axenic amastigotes did not show activity against intracellular amastigotes. This could be due to differences in cellular uptake or to the reported biochemical differences between the two amastigote forms (Alcolea et al., 2014, 2010; Rochette et al., 2009).

Interestingly, a high proportion of compounds (14 out of 16) showed activity against intracellular amastigotes but not against axenic amastigotes (Table 1 and Fig 6). This discrepancy could be due to the different threshold applied for considering positives, but the number of examples suggests a more probable host-cell-dependent mechanism of action (De Muylder et al., 2011). These results and the subtle differences between mammalian and parasitic tubulins suggest the possibility of an action on the host tubulin at sub-cytotoxic concentrations, which has been previously shown in the treatment of eukaryotic cells with tubulin inhibitors, contributing to the antileishmanial activity. The well-known capability of tubulins of different origins, specifically mammalian and leishmanial, to co-assemble and exchange dimer subunits would support the possibility of a tubulin-mix based mechanism (Montecinos-Franjola et al., 2019). Aside from representatives of the series (Fig 3 and Table 1) active against axenic amastigotes (**138** and **183** of the 2,5-dimethoxyphenyl series, **242** of the 3,5-dimethoxyphenyl, and **316** and **320** of the 6-methoxy-3-pyridyl series), new structural types of phenyl sulfonamides of 3,4-dimethoxyanilines (**26**, **35**, **117**, **124**, and **132**, that also includes **334** active against axenic amastigotes), or 3-carboxy-5-aminoanilines (**84A**, **90**, and **147**) are active against intracellular parasites herein, together with the triaryl **63B**.

3.8 Docking studies

To obtain insights into the binding of the active compounds to tubulin we have performed flexible docking studies with mammalian and leishmanial tubulins. The flexibility of the protein was accounted for by using several structures with different binding-site configurations. For the mammalian proteins, we used

50 X-Ray crystal structures of complexes with different ligands bound to the colchicine domain available in the Protein Data Bank and 5 more representative models from a molecular dynamics simulation, as previously described (Álvarez et al., 2013). For the leishmanial tubulin, 5 homology models were generated with Modeler (Šali and Blundell, 1993) for each unique combination of amino acids in the colchicine site present in the leishmanial sequences retrieved from UniProt. As a result, 25 models of leishmanial tubulin were used for the docking studies. The docking studies were performed with two frequently used docking programs that use very different scoring functions. For each ligand, several thousand poses were generated bound to each of the protein sets. The poses were automatically assigned to the occupied zones (A-D) of the binding domain, and the poses with the best consensus scores of the two programs were selected as the binding poses (Fig 7) (Supp Mat Table 2).

Most ligands bind in similar ways to the mammalian and the leishmanial protein, occupying sites A and B (Fig 7), and even the apparently large for a colchicine-site ligand **276B** binds similarly to ABT-751 (Supplemmental Fig SF1). The phenyl rings with the larger substituents are always located in zone 2, while the other ring binds at zone 1. The size reduction from the trimethoxyphenyl ring of combretastatin A-4 to those here employed (2,5- (**129**), 3,4- (**124**), and 3,5-dimethoxyphenyl and 3-carboxy-5-aminophenyl (**84**)) are a better fit for the smaller and more polar zone 2 of the parasite orthologs. For the sulfonamides with a 6-methoxy-3-pyridyl ring, such as **320**, the pyridine ring is in zone 1, thus confirming the importance of filling the available space in zone 2. The unoccupied space left by the smaller rings in zone 2 is responsible for the selectivity of the ligands, apparently associated with a reduction in the affinity for the host protein. The similar binding to the tubulin of the host and the parasite is consistent with the observed host-cell dependent action.

The compounds stack an aromatic ring at zone 1 between Asn258β of helix H8 and the sidechain methylenes of Ser316β and Lys or Arg352β of sheets S8 and S9, respectively, making additional hydrophobic contacts along the ring plane with the sidechains of Val181α, Leu255β, Met259β, and Thr314β. The other aromatic ring is inserted edgewise towards the surface of sheets S8 and S9 boxed between the sidechains of Ser316β and Val318 of the first and Lys or Arg352β, and Ser354β of the second,

overlaid by helices H7 and H8 and the loop between them and interacting with the sidechains of Cys241β, Leu242β, Leu248β, Ala250β, and Leu255β. These residues are highly conserved amongst the different species of *Leishmania* and therefore, the activity of the compounds might extend to other members of the genus.

4. Conclusions

A focused library of diarylsulfonamides has been designed to target the colchicine site of leishmanial but not mammalian tubulin, based on known structure-activity relationships (SAR), on the differences in amino acids between the two organisms, and the presumed favorable cost, stability, and solubility profiles. 350 new sulfonamides have been synthesized, characterized, and shown to have adequate solubility, stability, and cost compared with current antileishmanial treatments. To find new compounds against the ZVL-causing agent *L. infantum,* the compound library was evaluated *in vitro* against different parasite life cycle stages and using a human host cell line. None of them showed activity against promastigotes. However, 8 sulfonamides **129, 204, 332, 326B, 275, 279, 334,** and **276B** showed favorable activity profiles against axenic amastigotes, with IC_{50} values between 0.9 and 12 µM, similar or even better than the reference drug miltefosine. The structural requirements for activity against axenic amastigotes include dimethoxyphenyl rings substituted at the 2,5-, 2,3- or 3,4-position, not being very different from the requirements for interaction with human tubulin because 50 % were also cytotoxic against cancer human cell lines. 157 compounds lacking cytotoxic activity were assayed in intracellular amastigotes. As a result, 16 severely reduced the number of axenic amastigotes found in treated infected macrophages compared to the untreated controls. The high success rate of 10 % validates the design approach in a phenotypic assay reproducing the clinically relevant form in the mammalian host. Sulfonamides **129** and **276B** efficiently interrupted the course of the U937 macrophage infection in the micromolar range and constitute an interesting point for the further development of new antileishmanial drugs with a different target than those of the drugs in the clinic or clinical trials, that might be favorable for combination therapies. 14 of the 22 new sulfonamides active against intracellular amastigotes lacked activity against axenic amastigotes, thus indicating a host-dependent mechanism of action devoid of toxicity which could

be an advantage as an added barrier to the development of resistance by the parasite. The consistency of the biological activity found is reinforced by clustering of the active compounds in five chemical classes that also provide preliminary structure-activity relationships for further development. Molecular docking experiments support binding to the colchicine site of tubulin of the parasites, provide good agreement with the structure-activity relationship trends and suggest that the activity might extend to other *Leishmania* species. The appropriate combination of accessibility, antileishmanial activity, mechanism, parasite selectivity, and favorable solubility properties make the sulfonamides presented here a promising choice for the development of *Leishmania* infection animal model proof-of-concept assay as the next step.

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Legends to Figures

Fig 1. The tubulin colchicine site and differences between mammals and *Leishmanias*. (**A**) Chemical structure of combretastatin A-4 with the interaction zones indicated by colored rectangles. (**B**) Amino acid substitutions in trypanosomatids compared to mammals. (**C**) Ribbon model of the tubulin dimer with combretastatin A-4 bound in the colchicine site, indicated by a blue rectangle. (**D**) Detail of the colchicine-binding site with the three binding zones indicated as colored volumes and with the amino acids of the *Leishmania* sequences which vary from humans shown.

Fig 2. General structure of diarylsulfonamide library. General chemical synthetic route for the chemical library of 350 diarylsulfonamides: (a) Pyridine, CH_2Cl_2, rt, 1 h. (b) X-Halogen, K_2CO_3, DMF, rt, 12 h. (**B**) Some of the most frequent modifications carried out in aromatic ring B (Ar_B) and/or aromatic ring A (Ar_A) substituents. Structural variations in the library and main functional group modifications: (c) H_2, Pd/C, EtOAc, rt, 48 h. (d) p-formaldehyde, $NaBH_3CN$, AcOH, MeOH, reflux, 2 h. (e) R-Halogen, CH_2Cl_2, rt, 1-12 h. (f) Formic acid, CH_2Cl_2, rt, 24 h. (g) Trichloroacetic acid, $NaBH_4$, THF, rt, 12 h. (h) Acetic anhydride, CH_2Cl_2, rt, 30 min. (i) 1) $NH_2OH{\cdot}HCl$, pyridine, MeOH, reflux 24 h. 2) Acetic anhydride, pyridine, reflux, 48 h. (j) R-OH, H_2SO_4, rt, 3 h.

Fig 3: Structures of the tested sulfonamides that showed leishmanicidal activity.

Fig 4: Dose-response study against *L. infantum* intracellular amastigotes of non-cytotoxic sulfonamides with axenic activity. (**A**) Intracellular amastigote number per infected macrophage in the range of concentrations 20-5 µM of non-cytotoxic compounds that showed activity against both, axenic and intracellular *L. infantum* amastigotes, **129** and **276B**. Untreated control cells were run in parallel. Results shown by Box Plot data analysis are representative of three independent experiments. (**B**) Number of amastigotes accommodated per counted macrophage sorted in decreasing order in a representative sample of 200 macrophages for untreated sample (Control), **129** and **276B** treatments. (**C**) Percentage of infected U937 macrophages treated with sulfonamides **129** or **276B** in a range of concentrations 20-5 µM normalized to the percentage of infected macrophages in untreated controls (usually around 70%), taken as 100%.

Fig 5: Light microscopy image of *in vitro* macrophage infection and intracellular amastigote assay. Left: control cells without treatment, U937 macrophages infected with *L. infantum* amastigotes. Right: Treated cells with the compound **129** at 20 µM, infection clearly interrupted.

Fig 6: Dose-response study against *L. infantum* intracellular amastigotes of non-cytotoxic sulfonamides without axenic activity. (**A**) Effect on the *L. infantum* intracellular amastigotes activity in cells treated with non-cytotoxic and non-active sulfonamides on axenic amastigotes at 10 µM, but which showed a significant *in vitro* infection decrease at 20 µM. Data shown are representative of three independent experiments. (**B**) Box Plot data analysis of the number of intracellular amastigotes per infected cell in the range 20-5 µM of most potent non-cytotoxic sulfonamides without axenic activity that were more deeply studied, **320** and **63B**. (**C**) Percentage of infected treated cells normalized to the percentage of infected macrophages in untreated controls (usually around 70% and taken as 100%), at 20, 10, and 5 µM of compounds **320** and **63B**.

Fig 7: Docking poses for compounds 84, 124, 129, and 320. The left column corresponds to docking in the colchicine-site of mammalian proteins and the right column to homology models for the *Leishmania* orthologs. The proteins are shown as gray cartoons and relevant amino acid sidechains and the ligands are shown as sticks. The three zones of the colchicine-binding site are indicated as volumes colored in the same way as figure 1.

TABLES WITH LEGENDS

Table 1. Leishmanicidal and cytotoxic activities of sulfonamides that showed antileishmanial activity in any of the parasite stages.

Compound	*Leishmania infantum*			U937	HT-29	HeLa	MCF7
	Promastigotes	Axenic	Intracellular				
	10 µM	amastigotes	amastigotes	10 µM	1 µM	1 µM	1 µM

		IC_{50} (µM)	20 µM				
129	NA	0.93	A	NC	NC	NC	NC
138	NA	NA	A	NC	NC	NC	NC
204	NA	2.2	ND	C	C	C	C
183	NA	NA	A	NC	NC	NC	NC
332	NA	4.3	ND	C	C	C	C
326B	NA	8.2	ND	C	C	C	C
275	NA	8.1	ND	C	NC	NC	NC
242	NA	NA	A	NC	NC	NC	NC
320	NA	NA	A	NC	NC	NC	NC
316	NA	NA	A	NC	NC	NC	NC
279	NA	12	NA	NC	NC	NC	NC
334	NA	5.75	NA	NC	NC	NC	NC
124	NA	NA	A	NC	NC	NC	NC
132	NA	NA	A	NC	NC	NC	NC
117	NA	NA	A	NC	NC	NC	NC
35	NA	NA	A	NC	NC	NC	NC
26	NA	NA	A	NC	NC	NC	NC
84	NA	NA	A	NC	NC	NC	NC
90	NA	NA	A	NC	NC	NC	NC
147	NA	NA	A	NC	NC	NC	NC
63B	NA	NA	A	NC	NC	NC	NC
276B	NA	6.7	A	NC	NC	NC	NC
Miltefosine	A	4.4	A	NC	ND	ND	ND

Leishmanicidal activities of sulfonamides on *L. infantum* promastigotes and axenic amastigotes at 10 µM and IC_{50} calculation; on intracellular amastigotes at 20 µM and cytotoxicity on human tumor cell lines U937 at 10 µM and HT-29, HeLa and MCF7 at 1 µM. NA, NonActive at tested concentration. A, Active at tested concentration. NC, No Cytotoxic at tested concentration. C, Cytotoxic at tested concentration. ND, Not-determined. Assays are described in Materials and Methods.

Legends to Figures

Fig 1. The tubulin colchicine site and differences between mammals and *Leishmanias*. (**A**) Chemical structure of combretastatin A-4 with the interaction zones indicated by colored rectangles. (**B**) Amino acid substitutions in trypanosomatids compared to mammals. (**C**) Ribbon model of the tubulin dimer with combretastatin A-4 bound in the colchicine site, indicated by a blue rectangle. (**D**) Detail of the colchicine-binding site with the three binding zones indicated as colored volumes and with the amino acids of the *Leishmania* sequences which vary from humans shown.

Fig 2. General structure of diarylsulfonamide library. General chemical synthetic route for the chemical library of 350 diarylsulfonamides: (a) Pyridine, CH_2Cl_2, rt, 1 h. (b) X-Halogen, K_2CO_3, DMF, rt, 12 h. (**B**) Some of the most frequent modifications carried out in aromatic ring B (Ar_B) and/or aromatic ring A (Ar_A) substituents. Structural variations in the library and main functional group modifications: (c) H_2, Pd/C, EtOAc, rt, 48 h. (d) p-formaldehyde, $NaBH_3CN$, AcOH, MeOH, reflux, 2 h. (e) R-Halogen, CH_2Cl_2, rt, 1-12 h. (f) Formic acid, CH_2Cl_2, rt, 24 h. (g) Trichloroacetic acid, $NaBH_4$, THF, rt, 12 h. (h) Acetic anhydride, CH_2Cl_2, rt, 30 min. (i) 1) $NH_2OH{\cdot}HCl$, pyridine, MeOH, reflux 24 h. 2) Acetic anhydride, pyridine, reflux, 48 h. (j) R-OH, H_2SO_4, rt, 3 h.

Fig 3: Structures of the tested sulfonamides that showed leishmanicidal activity.

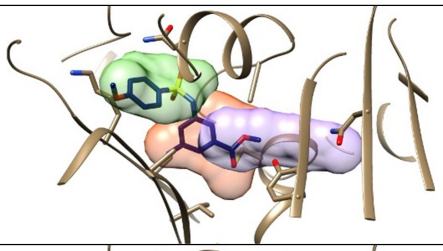
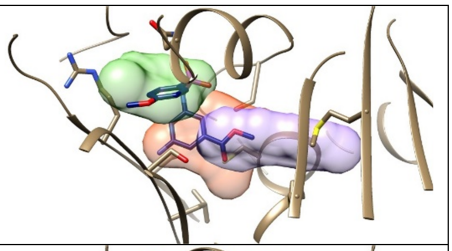
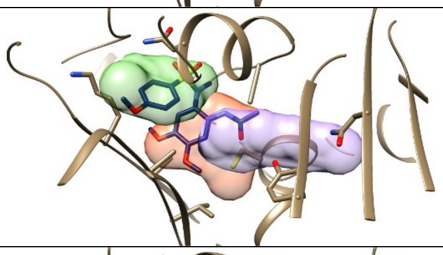
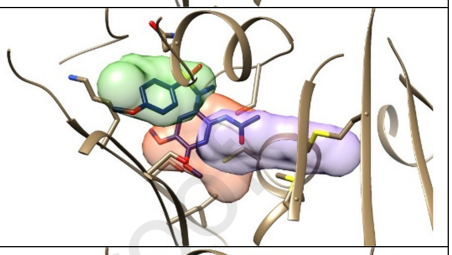
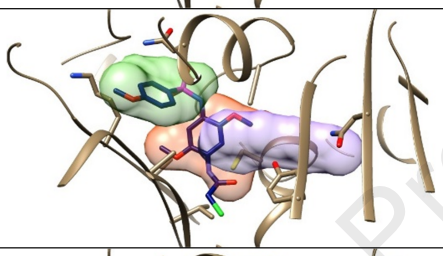
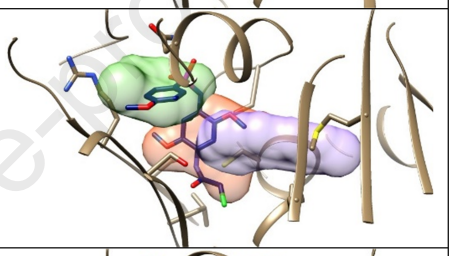
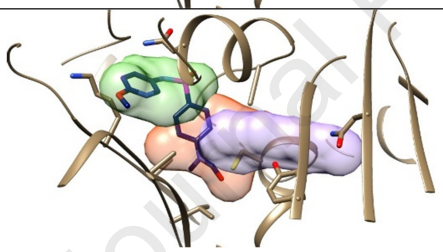
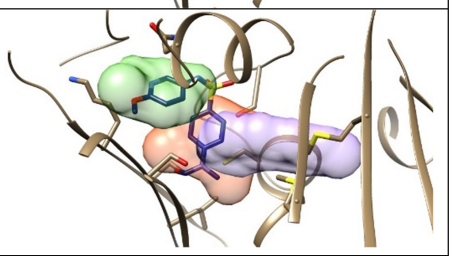
Fig 4: Dose-response study against *L. infantum* intracellular amastigotes of non-cytotoxic sulfonamides with axenic activity. (**A**) Intracellular amastigote number per infected macrophage in the range of concentrations 20-5 µM of non-cytotoxic compounds that showed activity against both, axenic and intracellular *L. infantum* amastigotes, **129** and **276B**. Untreated control cells were run in parallel. Results shown by Box Plot data analysis are representative of three independent experiments. (**B**) Number of amastigotes accommodated

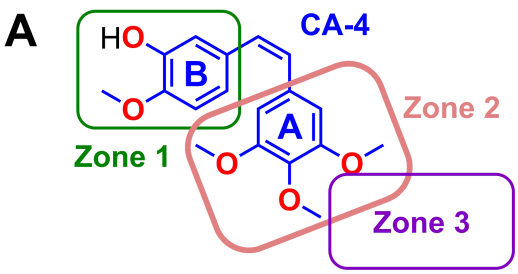
per counted macrophage sorted in decreasing order in a representative sample of 200 macrophages for untreated sample (Control), **129** and **276B** treatments. (**C**) Percentage of infected U937 macrophages treated with sulfonamides **129** or **276B** in a range of concentrations 20-5 µM normalized to the percentage of infected macrophages in untreated controls (usually around 70%), taken as 100%.

Fig 5: Light microscopy image of *in vitro* macrophage infection and intracellular amastigote assay. Left: control cells without treatment, U937 macrophages infected with *L. infantum* amastigotes. Right: Treated cells with the compound **129** at 20 µM, infection clearly interrupted.

Fig 6: Dose-response study against *L. infantum* intracellular amastigotes of non-cytotoxic sulfonamides without axenic activity. (**A**) Effect on the *L. infantum* intracellular amastigotes activity in cells treated with non-cytotoxic and non-active sulfonamides on axenic amastigotes at 10 µM, but which showed a significant *in vitro* infection decrease at 20 µM. Data shown are representative of three independent experiments. (**B**) Box Plot data analysis of the number of intracellular amastigotes per infected cell in the range 20-5 µM of most potent non-cytotoxic sulfonamides without axenic activity that were more deeply studied, **320** and **63B**. (**C**) Percentage of infected treated cells normalized to the percentage of infected macrophages in untreated controls (usually around 70% and taken as 100%), at 20, 10, and 5 µM of compounds **320** and **63B**.

Fig 7: Docking poses for compounds 84, 124, 129, and 320. The left column corresponds to docking in the colchicine-site of mammalian proteins and the right column to homology models for the *Leishmania* orthologs. The proteins are shown as gray cartoons and relevant amino acid sidechains and the ligands are shown as sticks. The three zones of the colchicine-binding site are indicated as volumes colored in the same way as figure 1.

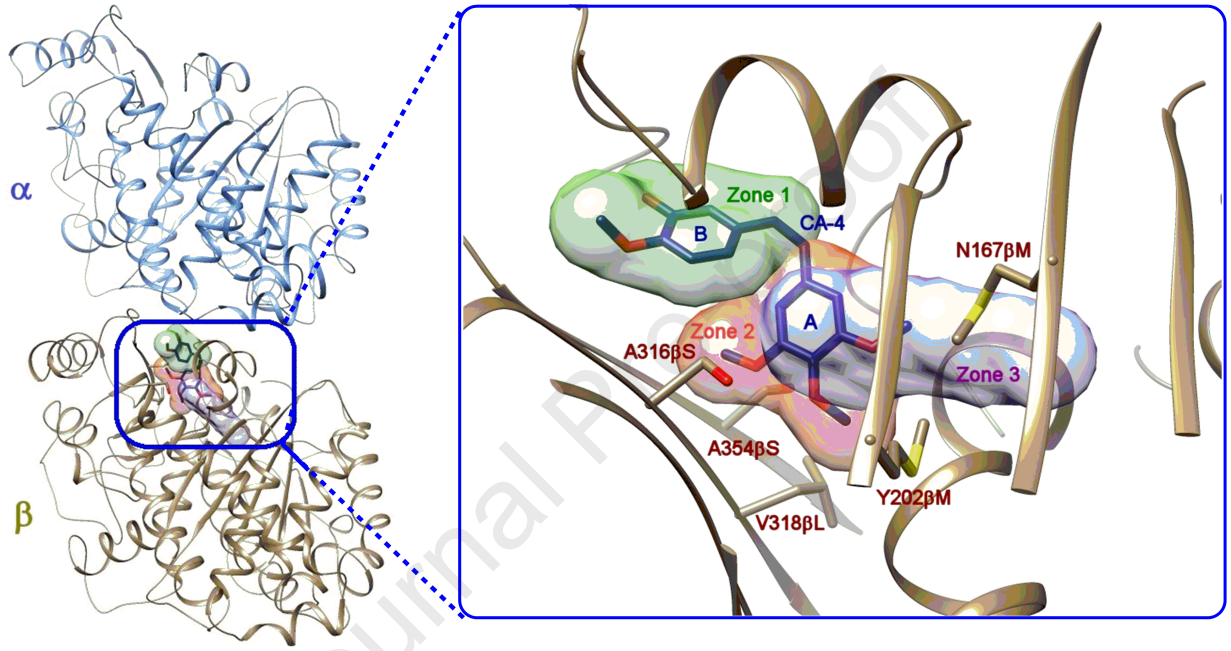
Comp	Mammal	Leishmania
84		
124		
129		
320		

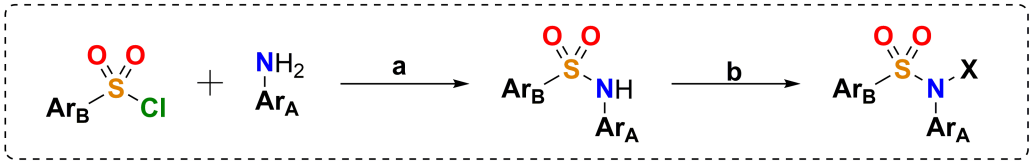


Combretastatin A-4

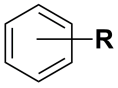
B

Zone 1	Zone 2	Zone 3
A316βS		N167βM/L
	A354βS	Y202βM
	V318βL	

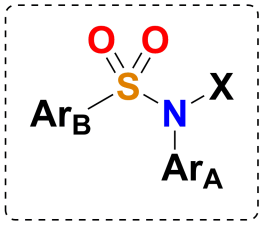




Ar_B:

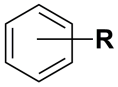


R: Alkoxy
NO_2
NR_1R_2: H
Alkyl
Acyl
Formyl

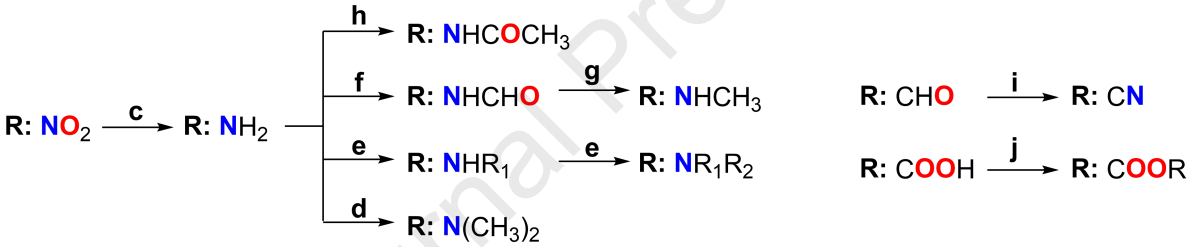


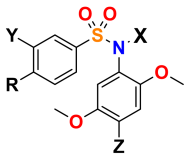
X: H
Alkyl
Halo-alkyl
Benzyl
CH_2CN
CH_2COOR: H
Alkyl

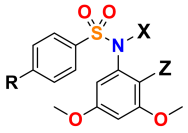
Ar_A:



R: Alkyl
Alkoxy
NO_2
NR_1R_2: H
Alkyl
Acyl
Formyl
SO_2Ph
CN
COOR: H
Alkyl
SO_2Ph
Pyridine

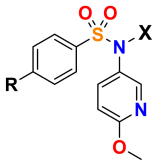




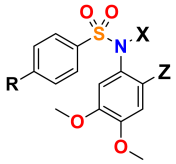


129: R = OCH_3 Z = $NHCOCH_2Cl$ X = H Y = H
138: R = OCH_3 Z = $NHCO(CH_2)_2Cl$ X = H Y = H
204: R = OCH_3 Z = Br X = CH_3 Y = NO_2
183: R = OCH_3 Z = H X = H Y = NO_2
332: R = $NHCH_3$ Z = Br X = Benzyl Y = H

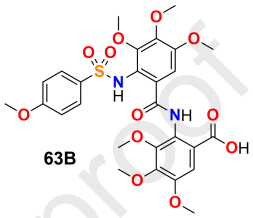
326B: R = OCH_3 Z = Br X = Benzyl
275: R = $N(CH_3)_2$ Z = H X = Benzyl
242: R = NO_2 Z = H X = H



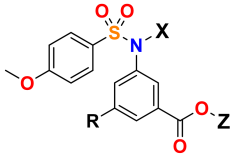
320: R = NCH_3CHO X = H
316: R = $NHCH_3$ X = H
279: R = OCH_3 X = Benzyl



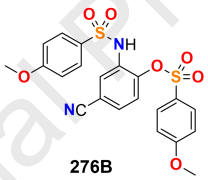
334: R = NO_2 Z = NO_2 X = H
124: R = OCH_3 Z = $NHCOCH_3$ X = CH_3
132: R = $N(CH_3)_2$ Z = NHCHO X = CH_3
117: R = $N(CH_3)_2$ Z, X = -N-N=N-
35: R = $N(CH_3)_2$ Z = NH_2 X = H
26: R = NH_2 Z = H X = H



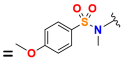
63B

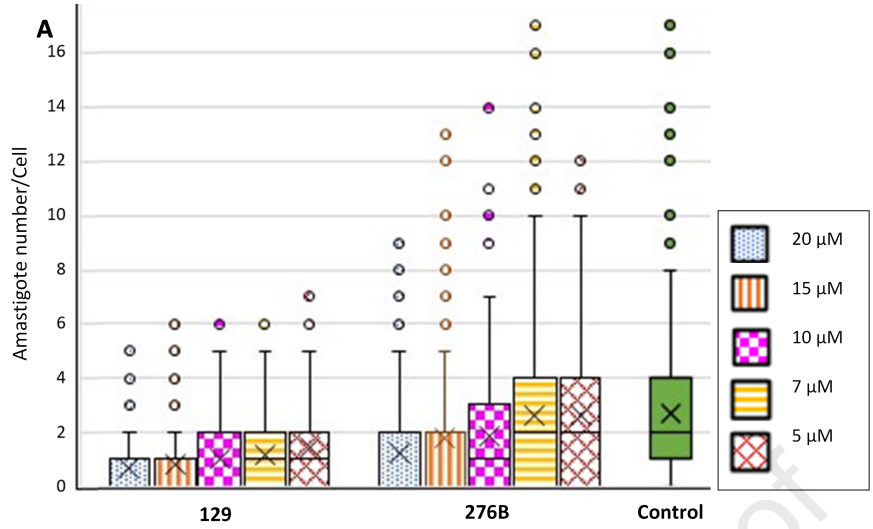


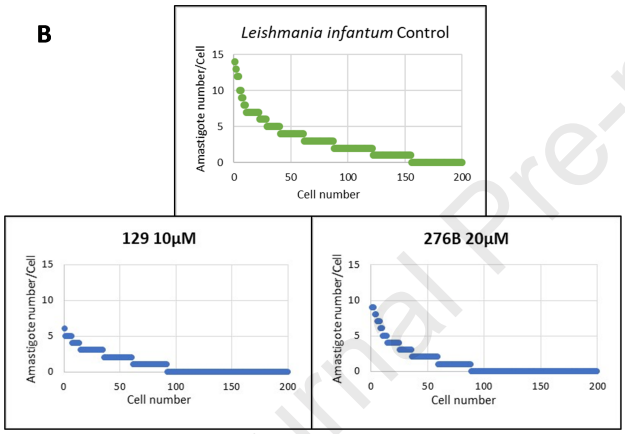
84A: R = NH_2 Z = CH_3 X = H
90: R = OCH_3 Z = H X = CH_3

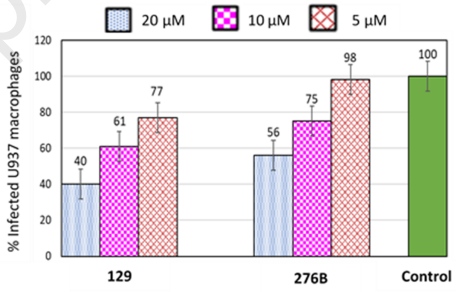


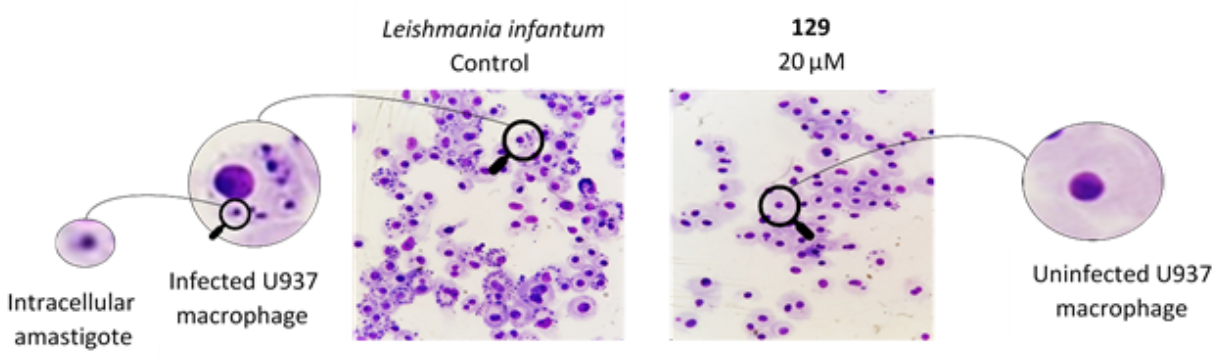
276B

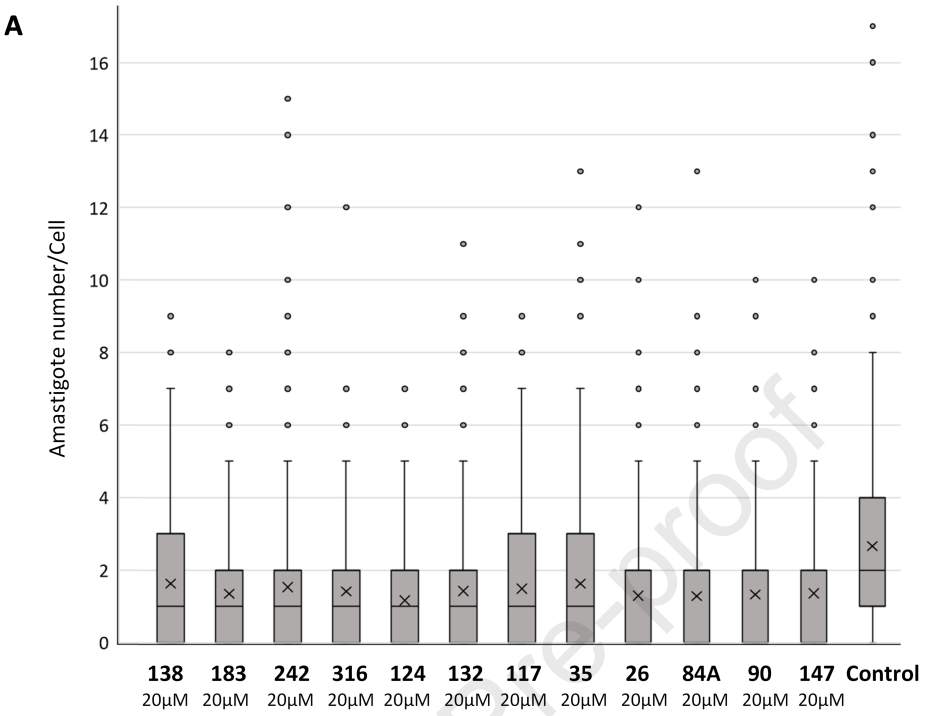
147: R =  Z = CH_3 X = CH_3

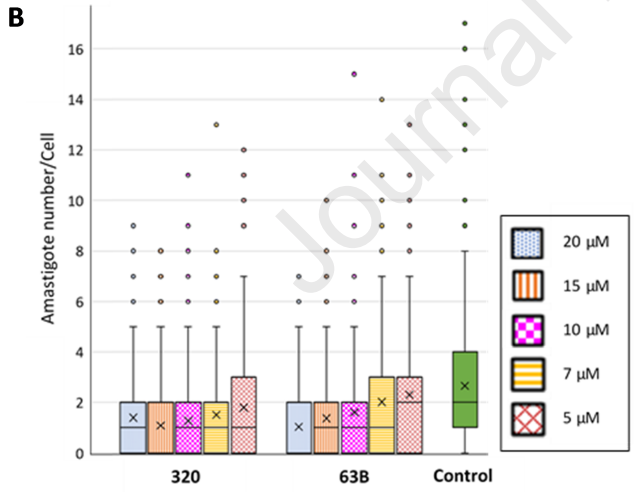


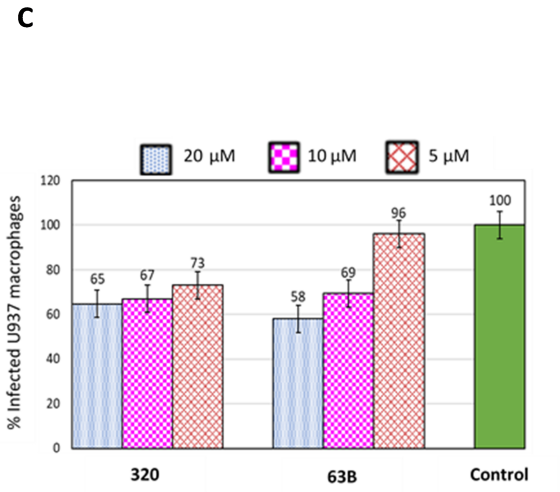












All authors declare no conflict of interest.