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N-[4-[Benzyloxy] benzyl]-benzenemethaneamines with High Biological Activity against Intracellular *Trypanosoma cruzi* and *Leishmania infantum* Amastigotes

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A B S T R A C T

Chagas disease and Leishmaniasis, caused respectively by *Trypanosoma cruzi* and *Leishmania spp.*, are neglected tropical diseases for which only unsatisfactory chemotherapeutic options are available. Therefore, there is still an urgent need to develop novel and improved pharmaceuticals for the treatment of these diseases. Inhibition of sterol biosynthesis has been proposed as a strategy for chemotherapy of *Trypanosoma spp.* and *Leishmania spp.*, because they mainly depend on endogenous ergosterol. Benzyl farnesyl amine has been proven to exhibit high inhibition of human squalene synthase (SQS), a key enzyme of cholesterol biosynthesis in man. Isosteric replacement of the farnesyl chain for benzyloxybenzyl substituent has been demonstrated to result in derivatives with stronger inhibition of human SQS and higher metabolic stability. These mimetics of benzyl farnesyl amine have never been tested for biological activity against *Trypanosoma cruzi* and *Leishmania infantum.* In this study, a small library of substituted *N*-[4-[benzyloxy] benzyl]-benzenemethaneamines was prepared by chemical synthesis and tested for biological activity against these pathogenic tropical protozoa. All compounds exhibited high activity against intracellular amastigotes of *Trypanosoma cruzi* and *Leishmania infantum*. *N*-[[4-[3′,4′-Dimethoxy]-benzyloxy]benzyl]-benzenemethaneamine hydrochloride **6 c** showed, with EC_{50} : 2.8 μM, the highest potency against *Trypanosoma cruzi*, comparable to the activity of the positive control benznidazole (EC_{50} : 3.0 μM), whereas (S)-α-methyl-*N*-[4-[benzyloxy]benzyl]-benzenemethaneamine hydrochloride **6 f** presented the most potent activity against *Leishmania infantum*, with EC_{50} : 7.7 μM. The compounds presented a high selectivity towards *Leishmania infantum* amastigotes. This finding demonstrates that structurally simple SQS inhibitors have a high potential in anti-Chagas and anti-Leishmaniasis drug design. It is of great importance for the development of novel, low cost antitrypanosomial and antileishmanial drugs in emerging countries.

Keywords: Benzyl Farnesyl Amine Mimetics, *Trypanosoma cruzi, Leishmania infantum,* Neglected Tropical Infectious Diseases Drug Discovery

A R T I C L E I N F O

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1. Introduction

Chagas disease is still a major health problem in tropical regions of Latin America. This disease is caused by infection with the protozoa *Trypanosoma cruzi.* The current drugs used in chemotherapy of Chagas disease, benznidazole and nifurtimox, are efficacious in the treatment of acute infections, but have only moderate efficacy in the treatment of chronic patients, beside being associated with severe side effects [1]. Leishmaniasis is a complex of diseases caused by the *Leishmania* parasites. The chemotherapy currently available is not only associated with severe toxicity, it also varies from different geographical locations and depends on the infecting species of *Leishmania* [2]. Beyond this, there is still a low economic interest to develop potent and cheap anti-parasitic drugs. Therefore, there is still an urgent necessity to develop novel pharmaceuticals against these tropical parasites.

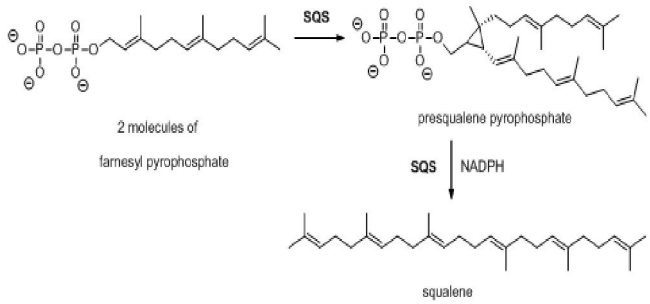


Figure 1: Condensation of two molecules of farnesyl pyrophosphate to squalene [3].

The enzymes of the ergosterol biosynthesis are attractive targets for development of specific treatment of Chagas disease and Leishmaniasis, because the trypanosomatid parasites require endogenous ergosterol and other 24-alkylated sterols for growth and survival and are unable to use the abundant supply of cholesterol present in mammalian host [3]. The relevance of sterol biosynthesis for *Trypanosoma cruzi* has been reviewed recently by Julio Urbina [4], while the essentiality of this pathway in *Leishmania* has been early demonstrated by Juliany C. F. Rodrigues, Julio Urbina and Wanderley de Souza [5]. The squalene synthase (SQS), which catalyzes the condensation of two moles of farnesyl pyrophosphate to squalene (Figure 1), the first committed step of the sterol pathway, became an attractive target [6,7] in Medicinal Chemistry, because SQS-inhibitors were expected to lower cholesterol levels in man without causing the same side effects observed for the well known HMG-CoA-reductase inhibitors [6,7]. Various aryl- and heteroarylquinuclidines were found to be strong inhibitors of protozoal *squalene synthase* (SQS) and growth of pathogenic tropical protozoa [3, 8-12] *Leishmania spp.*, *Trypanosoma spp.* and fungi like *Candida albicans*, *Candida tropicalis* and *Candida parapsilopsis.* [13].

Several ergosterol biosynthesis inhibitors exhibit high biological activity and low cytotoxicity. Julio Urbina demonstrated, that two known *human* SQS inhibitors with chiral heteroarylquinuclidine structure exhibit a high antitrypanosomal activity *in vitro* and have a high curative effect [14] on Chagas disease in a small animal model. Additionally, Juliany Rodrigues *et al.* have demonstrated the activity of two non-competitive inhibitors of *Leishmania amazonensis* squalene synthase against both forms of the parasite: promastigotes and intracellular amastigotes [12].
The activity was reported to be associated with the depletion of endogeneous sterols /accumulation of exogenous cholesterol [12].
A great variety of aryl- and heteroarylquinuclidines with high biological activity on pathogenic protozoa were reported by Julio Urbina, Juliany Cola F. Rodrigues and us [3, 8-13]. Recently we prepared a small library of chiral 3-[[(aryl)methyl]amino]- and 3-[[(heteroaryl)methyl]-amino]quinuclidines [15] with strong biological activity against *Trypanosoma cruzi* and investigated the effect of the stereochemistry on their antiparasitic activity [15].

In connection to our broad research program, to explore inhibitors of the sterol biosynthesis for their potential use to develop novel drugs against pathogenic tropical protozoa *Trypanosoma spp.* and *Leishmania spp.* we recently started a synthesis campaign to structurally more simple SQS inhibitors, mimetics of benzyl farnesyl amine. John A. Brinkman *et al.* have shown, that benzyl farnesyl amine [16] and mimetics, where the farnesyl side chain has been replaced for metabolically more stable benzyloxybenzyl substituents [16], were strong inhibitors of human squalene synthase (SQS). *N*-[4-[benzyloxy]benzyl]-benzenemethaneamine-type benzyl farnesyl amine mimetics have never been tested for biological activity against *Trypanosoma cruzi* and *Leishmania infantum.* Therefore we intended to synthesize a small library of *N*-[4-[benzyloxy]benzyl]-benzenemethaneamines and study their

biological activity against *Trypanosoma cruzi* and *Leishmania infantum* with the aim to obtain preliminary **s**tructure **a**ctivity **r**elationships (SAR) for this compound class, using a cell-based assay as a biological model for compound screening. Main focus of this SAR studies was on the effect of the electronic nature of the benzyloxy-substituent of *N*-[4-[benzyloxy]-benzyl]-benzenemethaneamines on the biological activity against pathogenic protozoa.

2. Materials and Methods

Chemistry

Compounds were purified by flash column chromatography or normal column chromatography on silica gel $F_{256,}$ Merck Darmstadt, 60 - 230 Mesh or 230 – 400 Mesh. Solvents were purchased by *Synth* (Productos para laboratory Ltd., São Paulo) or by Sigma-Aldrich in analytical grade quality and used without further purification. For thin layer chromatography TLC Silica gel 60 plates with fluorescence indicator F_{245} of Merck (Darmstadt) were utilized. Compounds with no UV absorption were detected by potassium permanganate/potassium carbonate reagent (2 % $KMnO_4$, 2 % K_2CO_3 in H_2O). ^{1}H-NMR- and ^{13}C-NMR- and DEPT-spectroscopy was performed on Bruker advanced 400, 500 and 600 MHz-NMR-spectrometers. IR-spectroscopy was performed on a FT-IR-spectrometer ABB Bomem 100. UV-spectroscopy was performed on an Agilent 8453 UV-spectrometer. The specific optical rotation was determined on a Perkin-Elmer Model 341 polarimeter. Melting points were determined on a melting-point-apparatus Fisher Scientific IA 9000. Mass spectroscopy and High Resolution Mass Spectroscopy was performed using an Agilent 6550 Accurate-Mass Q-TOF LC/MS system with Agilent Jet Stream technology for electrospray ionization. Total ion spectra were collected over a mass range of m/z 100−1,700 in positive modes. Anhydrous solvents were prepared by drying commercial solvents utilizing known standard methods (toluene was dried by distillation over Na, methylene chloride by distillation over CaH_2, methanol was dried by molecular sieve MS 4Å). Benzylalcohols **1 a-d** were bought by Sigma-Aldrich and used without further purification. p-Hydroxybenzaldehyde **3 a** and vanilline **3 b** were bought by Sigma Aldrich as well and used as received. Benzylamines **5 a, b** and benzylchloride **2 a** were bought by Sigma-Aldrich. Benzylchlorides **2 b-d** were prepared according to a procedure reported by T. Jaschinski *et al* [17], p-benzyloxybenzaldehydes **4 a-e** were prepared according to Jonathan B. Houze *et al* [18]. Benzylamines **6 a-g** were prepared by application of a reductive amination protocol of James C. Powers *et al* [19].

General Procedure for the Preparation of 6 a-g (Scheme 1)

A solution of aldehyde **4 a-e** (1 eq., 2 mmol) in absolute ethanol (5 mL) was added dropwise to the solution of benzyl amine **5 a** (or **5 b**) while stirring. The mixture was heated under reflux for 2 hours. $NaBH_4$ (4.2 mmol) was added, then it was heated under reflux for 2 more hours. The solvent was removed in vacuo. The residue was dissolved in dichloromethane and the solution was washed with 1 M NaOH, H_2O and dried with K_2SO_4. The solvent was removed under reduced pressure. The residue was dissolved in 1 mL of isopropanol, HCl (conc.) was added until pH 1 and the product precipitated by addition of 10-20 ml ether.

***N*-[4-[Benzyloxy]benzyl]-benzenemethaneamine hydrochloride 6 a**

Yield : 91 %, **M. P. :** 240,9 – 241,6 ºC, **^{1}H-NMR (CD_3OD, 400 MHz): δ [ppm] =** 4,205 (s, 2H); 4,24 (s, 2H); 5,156 (s, 2H); 7,08-7,13 (m, 2H); 7,30-7,54 (m, 12H) ppm. **^{13}C-NMR (CD_3OD, 400 MHz): δ [ppm] =** 51,53 (CH_2); 51,725 (CH_2); 71,0 (CH_2); 116,59 (CH); 124,33 (C); 128,15 (CH); 128,90 (CH); 129,48 (CH); 130,26 (CH); 130,63 (CH); 130,98 (CH); 132,41 (C); 132,62 (CH); 138,26 (C); 161,13 (C) ppm. **IR (ATR) :ν [cm^{-1}]** = 2927, 2788, 1612, 1559, 1515, 1422, 1377, 1244, 1177, 1041, 1012, 978, 920, 829, 780, 749, 695, 553, 508, 484 cm^{-1}. **UV (CH_3CN) : λ [nm]** = 204, 274 nm. **MS (ES)** : m/z (%) = 304.2 (100), 284.3 (1), 233.2 (1), 212.2 (1), 197.1 (3), 182.1 (2), 136.1 (1), 132.1 (4), 106.1 (1). **HRMS :** for ($C_{16}H_{14}N_2^+$) found = 304.19093, calculated = 304.1696.

***N*-[[4-[4´-Methoxy]-benzyloxy]benzyl]benzene-methaneamine hydrochloride 6 b**

Yield : 63,1%, **M. P. :** 212,6 – 213,1 ºC, **^{1}H-NMR (CD_3OD, 400 MHz) : δ [ppm] =** 3,82 (s, 3H); 4,20 (s, 2H); 4,24 (s, 2H); 5,08 (s, 2H); 6,92-6,96 (m, 2H); 7,07-7,11 (m, 2H); 7,36-7,39 (m, 2H); 7,41-7,45 (m, 2H); 7,47-7,52 (m, 5H) ppm. **^{13}C- NMR (CD_3OD, 400 MHz) : δ [ppm] =** 50,29 (CH_2); 50,45 (CH_2); 54,33 (CH_3); 69,51 (CH_2); 113,43 (CH); 115,32 (CH); 122,95 (C); 128,56 (CH); 128,86 (C); 128,91 (CH); 129,32 (CH); 129,60 (CH); 131,09 (C); 131,20 (CH); 158,72 (C); 159,91 (C) ppm. **IR (ATR): ν [cm^{-1}] =** 1612, 1513, 1429, 1379, 1239, 1176, 1026, 1004, 982, 810, 743, 702, 602, 546, 531, 492 cm^{-1}. **UV (CH_3CN): λ [nm]** = 203, 228, 274 nm. **MS (ES)**: m/z (%) = 334.1 (1), 122 (1), 121 (100), 106 (1). **HRMS:** for ($C_{16}H_{14}N_2^+$) found = 334.17977, calculated = 334.1802.

***N*-[[4-[3´, 4´-Dimethoxy]-benzyloxy] benzyl]-benzenemethaneamine hydrochloride 6 c**

Yield : 71%, **M. P. :** 182,4 – 184,1 ºC, **^{1}H-NMR (CD_3OD, 400 MHz): δ [ppm] =** 3,83 (s, 6H); 4,18 (s, 2H); 4,22 (s, 2H); 5,06 (s,2H); 6,93-7,09 (m, 4H); 7,41-7,51 (m, 8H) ppm. **^{13}C- NMR (CD_3OD, 400 MHz): δ [ppm] =** 52,28 (CH_2); 52,46 (CH_2); 57,15 ($2CH_3$); 71,71 (CH_2); 113,48 (CH); 113,52 (CH); 117,38 (CH); 122,35 (CH); 125,02 (C); 130,64 (C); 130,96 (CH); 131,33 (CH). 131,69 (CH); 133,14 (C); 133,36 (CH); 151,05 (C); 151,30 (C); 161,93 (C) ppm. **IR (ATR): ν [cm^{-1}] =** 2924, 2784, 2722, 1608, 1513, 1457, 1420, 1377, 1235, 1179, 1140, 1025, 978, 866, 829, 807, 747, 695, 553 cm^{-1}. **UV (CH_3CN): λ [nm]** = 204, 228, 277, 359 nm. **MS (ES)**: m/z (%) = 364.2 (1), 338.3 (1), 301.1 (1), 257.1 (1), 152.1 (1), 151.1 (100), 135.0 (1), 108.1 (1), 107.1 (1). **HRMS:** for ($C_{16}H_{14}N_2^+$) found = 364.1899, calculated = 364.1907

***N*-[[4-[3´,4´,5´-Trimethoxy]-benzyloxy]benzyl]-benzenemethaneamine hydrochloride 6 d**

Yield : 72 %, **M. P. :** 211,4 – 213,2 ºC, **^{1}H-NMR (CD_3OD, 400 MHz) : δ [ppm] =** 3,78 (s, 3H); 3,86 (s, 6H); 4,21 (s,

2H); 4,24 (s, 2H); 5,11 (s, 2H); 6,78 (s, 2H); 7,09-7,13 (m, 2H); 7,44-7,54 (m, 7H) ppm.

^{13}C- NMR (CD_3OD, 400 MHz) : δ [ppm] = 50,30 (CH_2); 50,48 (CH_2); 55,33 ($3CH_3$); 59,83 (C); 69,80 (CH_2); 115,34 (CH); 123,19 (C); 128,60 (CH); 128,93 (CH); 129,304 (CH); 129,66 (CH); 131,11 (C); 131,299 (CH); 133,01 (C); 153,31 (C); 159,75 (C) ppm. **IR (ATR) :ν [cm^{-1}] =** 2931, 2786, 1591, 1513, 1459, 1423, 1381, 1332, 1235, 1177, 1127, 1000, 982, 829, 810, 745, 695, 508 cm^{-1}. **UV (CH_3CN): λ [nm]** = 207, 275 nm. **MS (ES)** : m/z (%) = 394.2 (1), 310.1 (1), 246.2 (1), 182.1 (1), 181.1 (100), 148.0 (1), 107.9 (1). **HRMS : for** ($C_{16}H_{14}N_2^+$) found = 394.2003, calculated = 394.2013

***N*-[[3-Methoxy-4-benzyloxy] benzyl]benzene-methaneamine hydrochloride 6 e**

Yield : 65%, **Melting Point :** 112,1 – 113,6 ºC, **^{1}H-NMR (CD_3OD, 400 MHz) : δ [ppm] =** 3,92 (s, 3H); 4,21 (s, 2H); 4,25 (s, 2H); 5,17 (s, 2H); 7,01-7,18 (m, 3H); 7,31-7,41 (m, 3H); 7,43-7,54 (m, 7H) ppm.

^{13}C- NMR (CD_3OD, 400 MHz) : δ [ppm] = 52,77 (CH_2); 52,86 (CH_2); 57,57 (CH_3); 72,87 (CH_2); 115,93 (CH); 116,57 (CH); 124,94 (CH); 126,13 (C); 129,56 (CH); 129,89 (CH); 130,38 (CH); 131,19 (CH); 131,59 (CH); 131,96 (CH); 133,38 (C); 139,24 (C); 151,46 (C); 152,46 (C) ppm. **IR (ATR) : ν [cm^{-1}] =** 2778, 1517, 1459, 1425, 1256, 1224, 1146, 1023, 808, 734, 695, 510, 482 cm^{-1}. **UV (CH_3CN): λ [nm]** = 204, 280 nm. **MS (ES)** : m/z (%) = 334.7 (23), 333 (28), 315 (6), 301.1 (30), 288.2 (10), 241.2 (25), 227.1 (43), 195.1 (100), 167.1 (43), 135 (16), 132.1 (47), 106.1 (6). **HRMS :** for ($C_{16}H_{14}N_2^+$) found = 334.17977 calculated = 334.1802.

(R)-(+)-α-Methyl-*N*-[4-[benzyloxy]benzyl]-benzenemethaneamine hydrochloride 6 f

Yield : 68,1%, **M. P. :** 126,4 - 129,2 ºC, **$[\alpha]^D_{20}$ =** + 16,80 (c = 1 in CH_3CN), **^{1}H-NMR (CD_3OD, 400 MHz): δ [ppm] =** 1,69 (d, *J* = 7Hz, 3H); 3,85 (d, *J* = 13Hz, 1H); 4,06 (d, *J* = 13Hz, 1H); 4,41 (q, *J* = 7Hz, 1H); 5,12 (s, 2H); 7,02-7,07 (m, 2H); 7,27-7,38 (m, 5H); 7,39-7,52 (m, 7H) ppm. **^{13}C-NMR (CD_3OD, 400 MHz): δ [ppm] =** 20,6 (CH_3); 51,0 (CH_2); 60,3 (CH); 71,9 (CH_2); 117,5 (CH); 125,3 (C); 129,36 (CH); 129,51 (CH); 129,77 (CH); 130,33 (CH); 131,43 (CH); 131,56 (CH); 133,29 (CH); 138,49 (C); 139,12 (C); 161,94 (C) ppm. **IR (ATR):ν [cm^{-1}]** = 2937, 2907, 2750, 2441, 1615, 1589, 1518, 1498, 1451, 1386, 1246, 1213, 1187, 1086, 1025, 989, 941, 864, 810, 762, 745, 698, 557, 514, 452 cm^{-1}. **UV (CH_3CN): λ [nm]** = 203, 220, 275 nm. **MS (ES)** : m/z (%) = 318.2 (4), 220.1 (1), 198.1 (1), 197.1 (100), 169.1 (4), 141.1 (1), 132.1 (10), 120.1 (2), 105.1 (26). **HRMS :** for ($C_{16}H_{14}N_2^+$) found = 318.18506, calculated = 318.1852

(R)-(+)-α-Methyl-*N*-[[4-[4′-methoxy]benzyloxy]benzyl]-benzenemethanamine hydrochloride 6 g

Yield : 56%, **M. P. :** 173,3 - 174,6 ºC, **$[\alpha]^D_{20}$ =** + 19,00 (c = 1 in CH_3CN), **^{1}H-NMR (CD_3OD, 400 MHz): δ [ppm] =** 1,73 (d, *J* = 7Hz, 3H); 3,81 (s, 3H); 3,87 (d, *J* = 13Hz, 1H); 4,08 (d, *J* = 13Hz, 1H); 4,44 (q, *J* = 7Hz, 1H); 5,05 (s, 2H); 6,91-6,96 (m, 2H); 7,04-7,08 (m, 2H); 7,31-7,39 (m, 4H); 7,48-7,56 (m, 5H) ppm. **^{13}C- NMR (CD_3OD, 400 MHz): δ [ppm] =** 20,8 (CH_3); 51,1 (CH_2); 56,7 (CH); 60,4 (CH_3); 71,9 (CH_2); 115,9 (CH); 117,6 (CH); 125,3 (C); 129,60 (CH); 131,1 (C); 131,21 (CH); 131,51 (CH); 131,64 (CH); 133,37 (CH); 138,58 (C); 161,91 (C); 162,07 (C) ppm. **IR(ATR) : ν [cm^{-1}]** = 2933, 2704, 2605, 2419, 1612, 1584, 1515, 1457, 1425, 1384, 1308, 1246, 1176, 1086, 1036, 1010, 982, 870, 816, 796, 773,747, 698, 602, 551, 516 cm^{-1}. **UV (CH_3CN) : λ [nm]** = 203, 228, 274 nm. **MS (ES):** m/z (%) = 348.2 (1), 122.1 (1), 121.1 (100), 120.1 (1), 107 (1), 105.1(1). **HRMS :** for ($C_{16}H_{14}N_2^+$) found = 348.19533, calculated = 348.1958.

Biology

The human host cell and parasite lines culture media (all from Hyclone) were supplemented with 10% heat inactivated fetal bovine serum (Gibco) and a solution of 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco). Each experiment was performed in duplicate (i.e. two independent experiments).

***In vitro* evaluation of activity against *Leishmania infantum* intracellular amastigotes**

THP-1 cells were plated onto black µClear 384-well tissue culture treated polystyrene plates in RPMI complete media containing 50 ng/mL PMA, and incubated for 48 h at 37 ºC and 5% CO_2. Then, *Leishmania infantum* promastigotes were added onto the assay plate. After 24 h of infection, negative controls (0.5% DMSO), positive controls - 10 µM amphotericin B – or the serially diluted compounds were manually added into the plate. Initially, compounds were tested at 25 µM, while selected hits were tested in dose-response (10-point, 2-fold-dilution starting at 50 µM) at an activity confirmatory screening. Assay plates were incubated for 72 h and processed subsequently for image acquisition at the Operetta High Content Screening system (Perkin Elmer).

***In vitro* evaluation of activity against *Trypanosoma cruzi* intracellular amastigotes**

The assay was performed as described by Carolina Moraes and collaborators [20]. For the single-concentration primary assay, the compounds were tested at 25 µM, while 100 µM was the highest concentration in the confirmatory dose-response assay (14 points, 2-fold-dilutions) and 400 µM was the highest concentration for reference compound benznidazole (Epichem). Assay plates were incubated for 96 h prior to image acquisition.

High content screening and image analysis

At the assays endpoint, plates were fixed with 4% PFA and stained with Draq5 (Biofocus). The multiparametric High Content Analysis yielded the quantification of host cells number, infection ratio and number of parasites per infected cell. Infection ratio as well as normalized activity were calculated as described [21]. Activity values were processed with the Graphpad Prism software, version 7, for generation of sigmoidal dose-response (variable slope) nonlinear curve fitting and determination of EC_{50} and CC_{50} values by interpolation.

3. Results and Discussion

Chemistry

Reaction of benzylalcohols **1 b-d** with 2 eq $SOCl_2$ in anhydrous ether at room temperature, according to a procedure recently prescribed by T. Jaschinski *et al* [17],

furnished the benzylchlorides **2 b-d** in high yields. The crude product was directly used in the next step. Alkylation of 4-hydroxybenzaldehydes **3 a, b** with benzylchlorides **2 a-d** in DMF with K_2CO_3 and NaI (cat.), according to Jonathan B. Houze *et al* [18] resulted in almost quantitative formation of **4 a-e**. *N*-[4-[Benzyloxy]benzyl]-benzene-methaneamines **6 a-g** were prepared by application of a reductive amination protocol of James C. Powers *et al* [19]. Thus a solution of 4-benzyloxybenzaldehydes **4 a-e** and benzylamines **5 a** (or **5 b**) in dry ethanol was heated to reflux for 2 hours. After that time, 2 eq sodium boranate was added and the mixture was heated additionally 2 hours to reflux. After removal of the solvents and inorganic salts the oily products were crystallized as hydrochlorides **6 a-g** by addition of i-PrOH, HCl and ether and obtained in a yield of 56-91 % as nice snow white crystals (Scheme 1).

Biology

High content assays [22] were used for both *Trypanosoma cruzi* and *Leishmania infantum* biological systems in order to evaluate the compounds antiparasitic activity against the intracellular replicative form amastigote. The use of a high throughput microscopic (high content screening) approach for image processing and data quantification delivers less biased and more reliable result, if compared to the laborious and tendentious manual counting methodology. Furthermore, this high content assay stands out due to the fact that compounds are tested against the disease-relevant stage of the protozoan parasites, the intracellular amastigotes, and enables simultaneous analysis of activity against host cells and parasites. Interestingly, all testing compounds presented relatively high antiparastic activity in the primary screening against *Trypanosoma cruzi* and *Leishmania infantum,* reaching at least 75% of activity at concentration of 25 μM (figure 2). Among the tested compounds, compound **6 g** presented the highest potency (i.e., low EC_{50} values) and efficacy (i.e., high maximum activity) against *Trypanosoma cruzi* and *Leishmania infantum* intracellular amastigotes (table 1 and figures 3 - 4), albeit it was only moderately selective toward the parasite in the case of *Trypanosoma cruzi* (S.I. < 10) (table 1 and figures 3 – 4). In fact, except for compound **6 c**, which presented higher selectivity, all the other tested compounds presented only moderate selectivity indexes in the U2OS-based *Trypanosoma cruzi* assay.

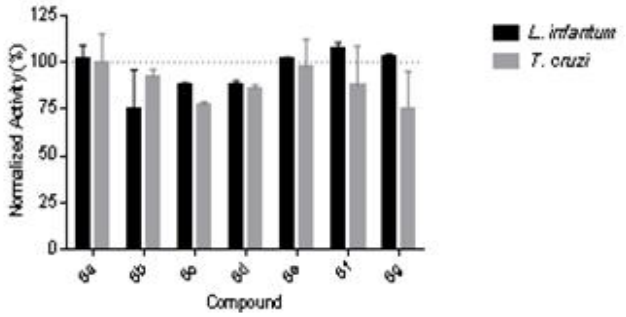


Figure 2: Activity of the tested compounds at 25 μM against *Trypanosoma cruzi* and *Leishmania infantum* intracellular amastigotes. Normalized antiparasitic activity indicates the inhibition of infection in relation to controls. Data are means and error bars represent standard deviations of two replicates.

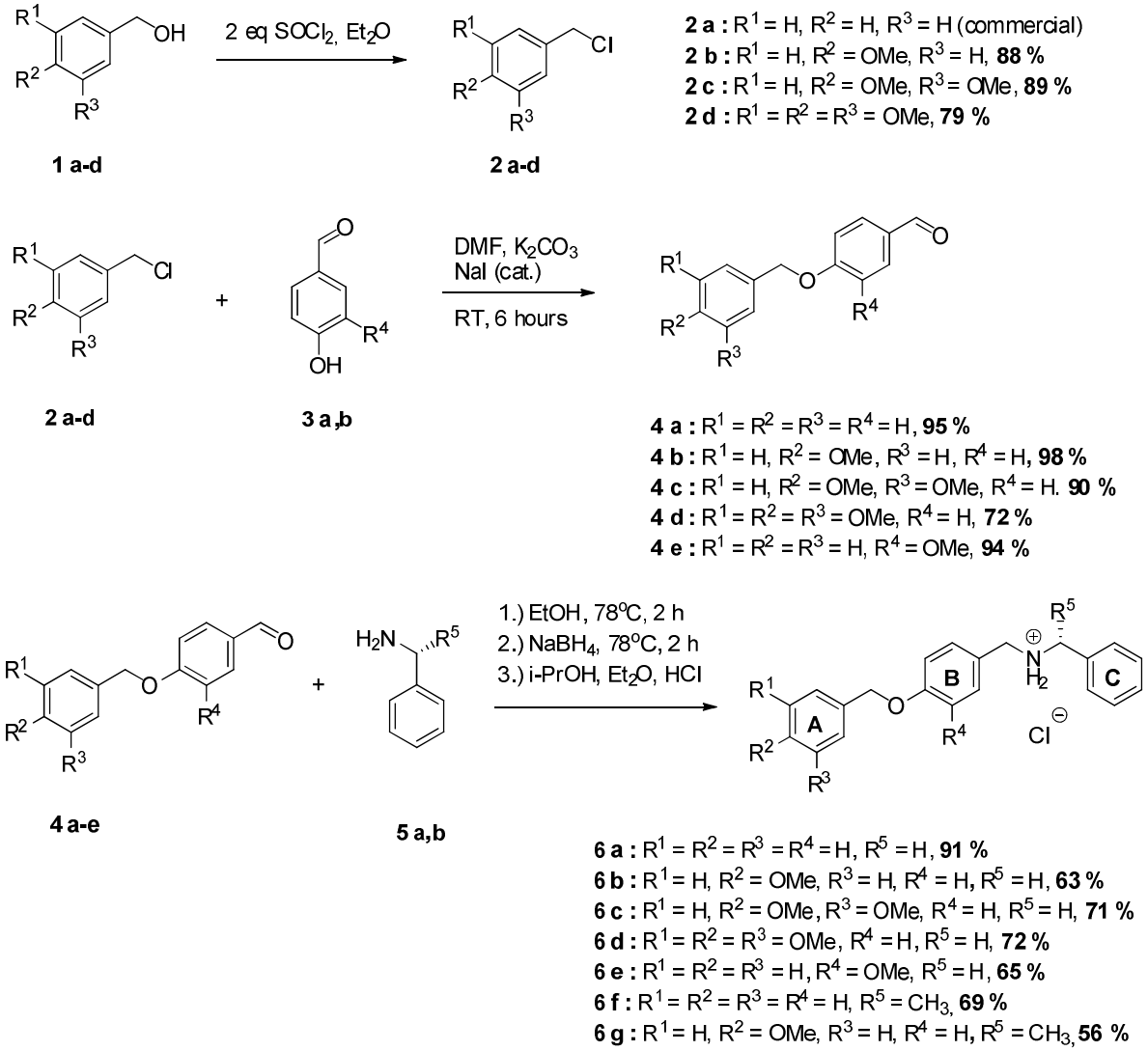
Medicinal Chemistry

All tested compounds presented relatively high antiparastic activity in the primary screening against *Trypanosoma cruzi* and *Leishmania infantum,* reaching at least 75% of activity at concentration of 25 μM (figure 2). **6 a** exhibits a moderate biological activity against *Trypanosoma cruzi* with EC_{50} = 7,1 μM and a low activity against *Leishmania infantum* with EC_{50} = 14,6 μM (Table 1). A OMe-substituent in 4-position of the A-ring leads to **6 b** with an almost twofold increase in activity against *Trypanosoma cruzi* (EC_{50} = 4,2 μM) and a non-significant increase in activity against *Leishmania infantum* (EC_{50} = 13,6 μM). The 3,4-dimethoxyderivative **6 c** shows a further significant increase in activity against *Trypanosoma cruzi* (EC_{50} = 2,8 μM) but a significantly lower activity against *Leishmania infantum* (EC_{50} = 17,9 μM). **6 c** is remarkably slightly more potent against *Trypanosoma cruzi* as known drug Benznidazol, whereas the toxicity of **6 c** is significantly higher. Derivative **6 d**, with three OMe-substituents in the A-ring shows a significantly lower activity against *Trypanosoma cruzi* (EC_{50} = 9,2 μM) and the activity against *Leishmania infantum* is slightly higher (EC_{50} = 15,7 μM) as it is observed for **6 c**. Compared to the unsubstituted compound **6 a** derivative **6 e** shows a slightly increased activity against *Trypanosoma cruzi* (EC_{50} = 6,7 μM) and against *Leishmania infantum* (EC_{50} = 12,3 μM). A OMe-substituent at 3´-position of the B-ring has obviously only a small effect on the antiprotozoal activity. Compared to unsubstituted compound **6 a** derivative **6 f** shows an almost twofold increase in activity against *Trypanosoma cruzi* (EC_{50} = 4,6 μM) and *Leishmania infantum* (EC_{50} = 7,8 μM). The sterically hindered Me-group at the benzyl-position neighboured to the C-ring may increase the metabolic stability of **6 f**. An additional OMe group in 4-position of the A-ring leads to **6 g** with a high biological activity against *Trypanosoma cruzi* (EC_{50} = 3,8 μM) and against *Leishmania infantum* (EC_{50} = 7,7 μM). A careful comparison of structures and antiprotozoal activities of all compounds tested in this study**,** suggest the preliminary conclusion, that a methyl-substituent at the benzyl-position neighboured to the C-ring exhibits a stronger effect on the biological activity against *Leishmania infantum*, whereas OMe-substituents in 3 and 4-position of the A-ring lead to a strong increase of the activity against *Trypanosoma cruzi* intracellular amastigotes. Enantiomers of chiral compounds **6 f** and **6 g** should be tested for antileishmanial and antitrypanosomial activity in order clarify the influence of the absolute configuration of their stereocenter on the biological activity against these pathogenic tropical protozoa.

4. Conclusion

In this study a small library of *N*-[4-[benzyloxy]benzyl]-benzenemethaneamines was prepared by chemical synthesis. The compounds were tested for biological activity against intracellular *Trypansoma cruzi* and *Leishmania infantum* amastigotes utilizing an innovative High Throughput/High Content (HT/HC) screening assay. All tested compounds presented relatively high antiprotozoal activity in the primary screening against *Trypanosoma cruzi* and *Leishmania infantum,* reaching at least 75% of activity at

concentration of 25 µM. *N*-[[4-[3′,4′-Dimethoxy]-benzyloxy] benzyl]-benzenemethaneamine hydrochloride **6 c** showed the highest biological activity against *Trypanosoma cruzi* (EC_{50} = 2,8 µM) and the lowest cytotoxicity (CC_{50} = 32,2 µM) to the host cell. The biological activity against *Trypanosoma cruzi* of **6 c** is significantly higher as the activity of known drug benznidazol (EC_{50} = 3,0 µM). Due to the low potency of benznidazol against chronic Chagas disease, it is, of course of high interest to test **6 c** in a small animal model of chronic Chagas disease. Chiral compounds (R)-(+)-α-methyl-*N*-[4-[benzyloxy]benzyl]-benzenemethaneamine hydrochloride **6 f** and (R)-(+)-α-methyl-*N*-[[4-[4′-methoxy] benzyloxy] benzyl]-benzenemethaneamine hydrochloride **6 g** exhibited the highest biological activities against intracellular *Leishmania infantum* amastigotes (**6 f :** EC_{50} = 7,8 µM, **6 f :** EC_{50} = 7,7 µM) and a high biological activity against intracellular *Trypanosoma cruzi* amastigotes (**6 f :** EC_{50} = 4,6 µM, **6 f :** EC_{50} = 3,8 µM), whereas their cytotoxicity was significantly higher (< 10 µM) in this host cell system. Preliminary **s**tructure **a**ctivity **r**elationships (SAR) suggest, that one or two OMe-substituents in 3 or 4 position of the A-ring of *N*-[4-[benzyloxy]benzyl]-benzenemethaneamine lead to a strong increase in activity against *Trypanosoma cruzi* and a non-significant change in activity against *Leishmania infantum*. A third OMe-substituent in the A-ring leads to a significant decrease in activity against *Trypanosoma cruzi* and a non-significant change in activity against *Leishmania infantum.* The α-methyl substituent in (R)-(+)-α-methyl-*N*-[4-[benzyloxy]benzyl]-benzenemethaneamine hydrochloride **6 f** and (R)-(+)-α-methyl-*N*-[[4-[4′-methoxy]-benzyl-oxy]-benzyl]-benzene-methaneamine hydrochloride **6 g** has a strong influence on the biological activity against *Leishmania infantum*. Omitting the α-methyl-substituent in **6 f** and **6 g** leads to an almost twofold decrease in activity against *Leishmania infantum.* This finding is of high potential to give strong impetus on research on the area of ergosterol biosynthesis inhibitors (EBI) and antitrypanosomal drug design. Although we strongly assume inhibition of squalene synthase (SQS) to be responsible for the biological activity of all compounds presented in this study, detailed investigations on the mode of action are currently done in collaboration with our partner groups in Campinas (LNBio) and Rio de Janeiro (UFRJ).



Scheme 1: Synthesis of *N*-[4-[Benzyloxy]benzyl]-benzenemethaneamines **6 a-g**

Table 1: Biological activity of **6 a-g** against *Trypanosoma cruzi* and *Leishmania infantum* intracellular Amastigotes

	Leishmania infantum				***Trypanosoma cruzi***			
	EC_{50} (µM)	CC_{50} (µM)	Max. Act. (%)	S.I	EC_{50} (µM)	CC_{50} (µM)	Max. Act. (%)	S.I
Amphotericin B	2.3 ± 0.5	41.4 ± 5.6	95.8	> 17.7	NT			
Benznidazole	NT				3.0 ± 0.8	ND	102.2	> 133.4
6 a	14.6 ± 2.5	ND	107.4	> 3.5	7.1 ± 3.4	21.1 ± 4.1	100.2	3.0
6 b	13.6 ± 3.3	ND	102.6	> 2.8	4.2 ± 1.4	25.8 ± 0.1	91.8	6.1
6 c	17.9 ± 0.5	ND	95.2	> 3.2	2.8 ± 0.4	32.2 ± 7.7	93.0	11.5
6 d	15.7 ± 0.1	ND	93.6	> 4.1	9.2 ± 0.5	27.2 ± 2.0	85.5	3.0
6 e	12.3 ± 2.0	ND	97.5	> 3.8	6.7 ± 2.0	16.7 ± 0.7	97.6	2.5
6 f	7.8 ± 2.8	ND	108.2	> 6.9	4.6 ± 1.5	16.6 ± 3.9	100.9	3.7
6 g	7.7 ± 0.7	ND	103.8	> 6.5	3.8 ± 2.0	21.9 ± 4.5	103.7	5.8

IC_{50} and CC_{50} values are expressed as averages ± standard deviations; ND, not determined; NT: not tested;. Max. Act.: maximum activity observed in the dose response- curve. S.I. selectivity index, a ratio between CC_{50} and EC_{50} whenever CC_{50} could not be calculated, S.I. is determined as a ratio between the highest compound concentration tested and EC_{50}. Ampho B: amphotericin B; Benz: benznidazole. n = 2 independent experiments.

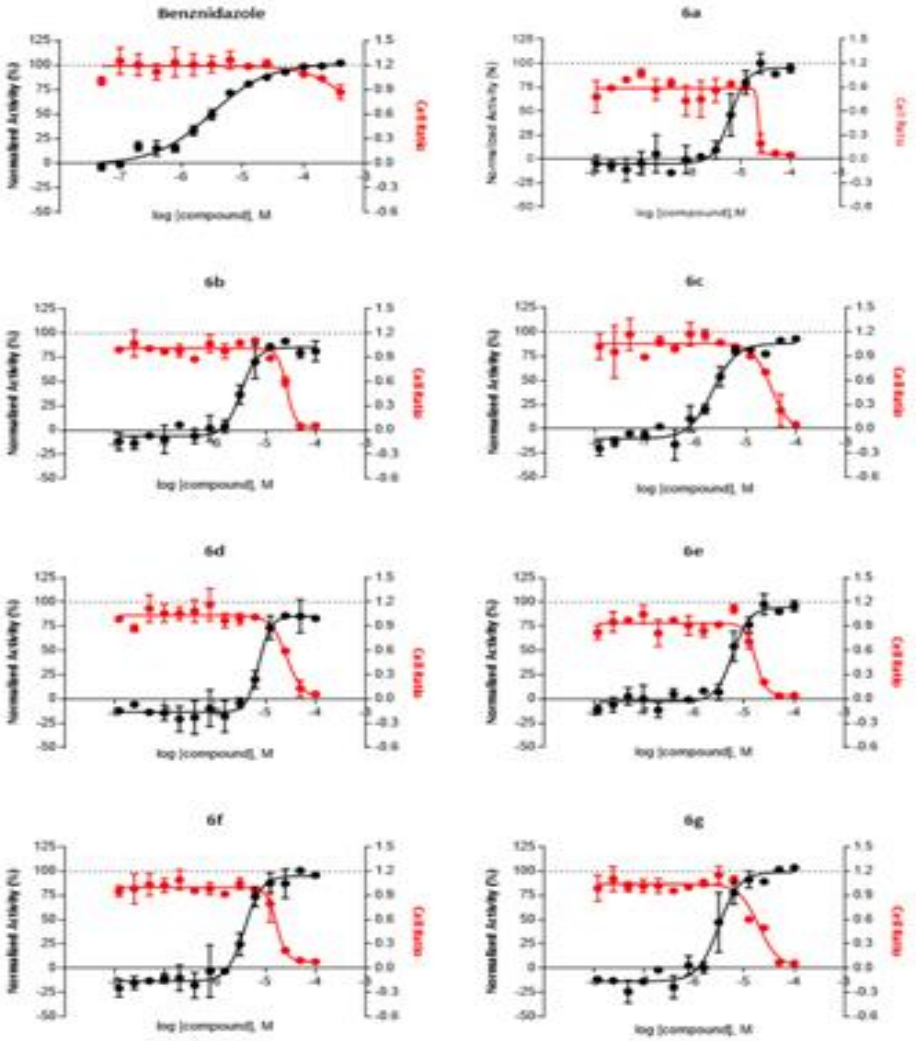


Figure 3: Dose-response curves for compounds after 96 h of drug exposure to U2OS cells infected with *Trypanosoma cruzi* Y strain. Data points are means and error bars represent standard deviations of two replicates. Black dots and curves represent data of compound activity whereas red dots and curves indicate the cell ratio parameter.

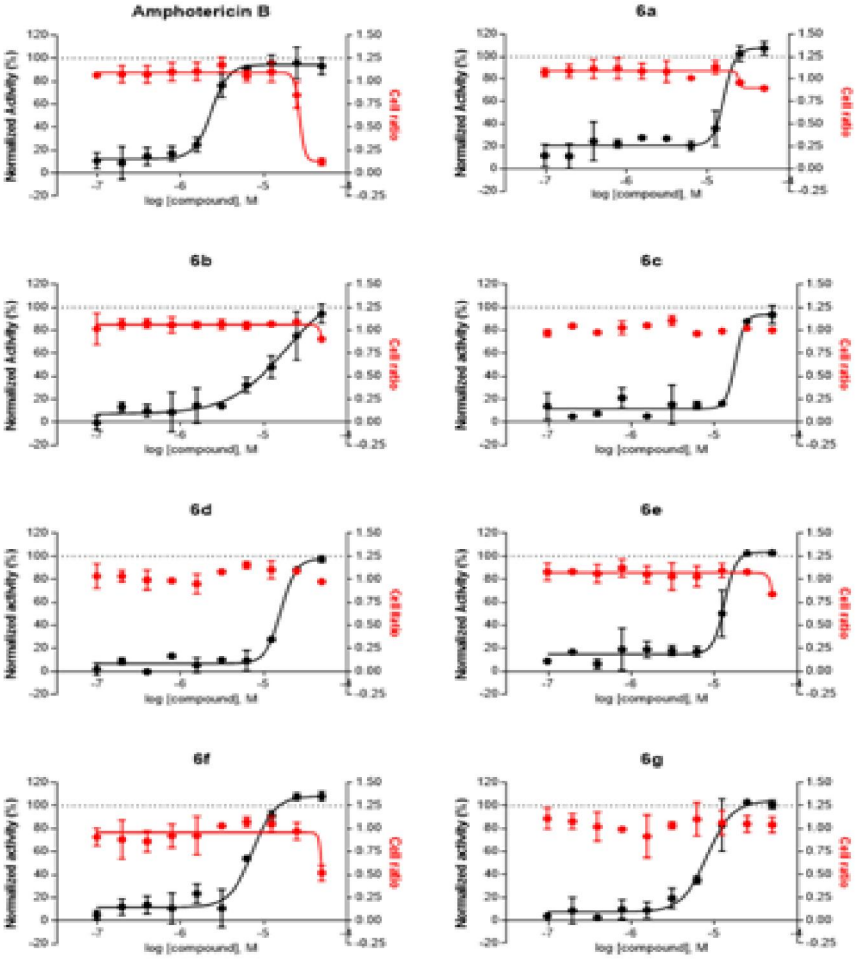


Figure 4: Dose-response curves for compounds after 48h of drug exposure to THP-1 cells infected with *Leishmania infantum* strain MHOM/BR/1972/BH46. Data points are means and error bars represent standard and curves indicate the cell ratio parameter.

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