

Parallel solution synthesis of lavendustin analogs as antileishmanial agents

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Dedicated to Dr. Manuel Gonzalez Sierra on the occasion of his 65th birthday

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Abstract

A concise parallel synthesis of 12 lavendustin analogs is described. Starting with differently substituted benzaldehydes, a two-step sequence involving reductive amination and *N*-benzylation accelerated by microwaves was performed to provide the compounds in excellent yields. The compounds have been assayed *in vitro* showing activity against *Leishmania donovani*, the causative agent of visceral leishmaniasis.

Keywords: Benzylamines, reductive amination, microwave, leishmaniasis

Introduction

Leishmaniasis is a parasitic disease caused by protozoan haemoflagellates of the genus *Leishmania*, which has more than 30 known species, with 20 of those species being pathogenic towards humans.¹ There are three main forms of the disease: cutaneous, muco-cutaneous and visceral, the latter being the most severe form, fatal in almost 100% of the cases if left untreated. Visceral leishmaniasis (VL) has major public health implications in the Indian subcontinent (Bangladesh, India and Nepal), East Africa (Ethiopia, Kenya and Sudan) and Latin America (Brazil). It has been estimated that this diseases has 500,000 cases per annum and affects mostly the poorest and most marginalized communities living in primarily rural areas. Of the annual

cases, 60% occur in about 109 districts of India, Bangladesh and Nepal, where about 150 million people are at risk of developing VL.² Visceral leishmaniasis is primarily caused by *Leishmania donovani* and affects the spleen, liver, lymph nodes and bone marrow.

Pentavalent antimonial compounds, sodium stibogluconate (Pentosan) and meglumine antimoniate (Glucantime) have been used for many decades as the main choice in antileishmanial chemotherapy.³ Some drawbacks associated with these drugs are their high toxicities, variable efficacy, intravenous administration in high dosages and an increasing number of reports of drug resistance, especially in endemic areas.⁴

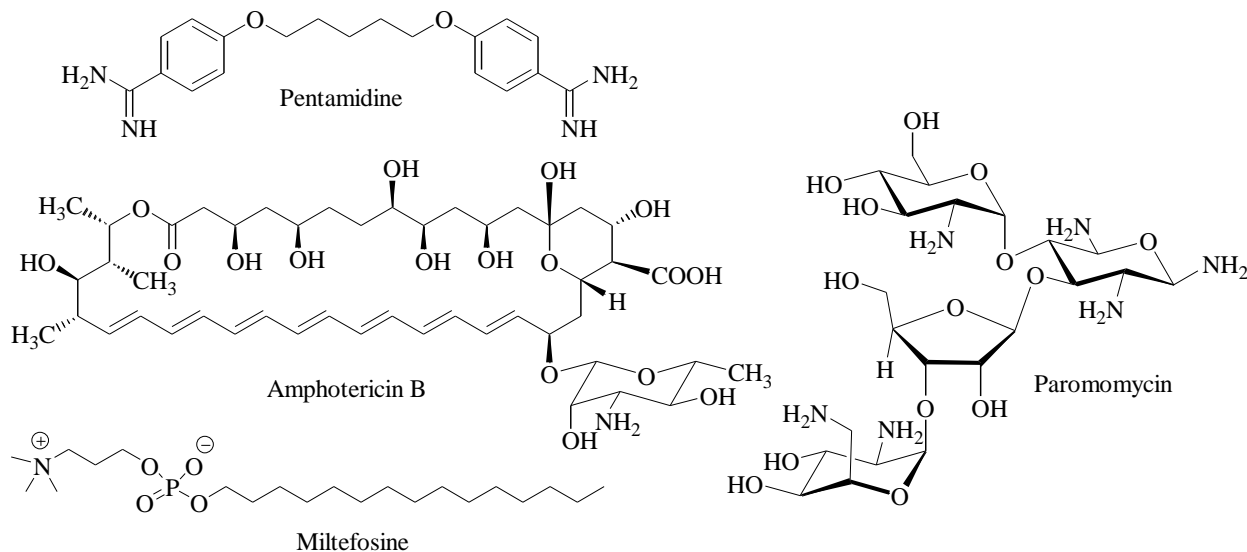


Figure 1. Antileishmanial drugs.

Recently, Pentamidine and Amphotericin B has been added as alternative drugs but, for different reasons, *i.e.* high prices and undesirable side effects and difficult availability, are limited.⁵ Miltefosine, a new drug for the treatment of visceral leishmaniasis went to Phase IV trials in public and private clinics in India few years ago,⁶ and has been recommended for visceral disease in India and in Ethiopia, and for the cutaneous form of the disease in Colombia and Bolivia.⁷ Paromomycin, was originally isolated from *Streptomyces krestomuceticus* in the 1950s, and studied as an antibiotic. This compound has recently passed a multi-center phase III study in India, demonstrating high efficacy, affordability, and presenting itself as a safe treatment.⁸ These results prompted the Drug Controller General of India (DCGI) to approve paromomycin for the treatment of visceral leishmaniasis.⁹ Despite the encouraging success obtained thus far, there is a long way to go yet in order to find new and improved drug candidates, to add to the arsenal of oral chemotherapeutics against leishmaniasis and other neglected parasitic diseases.¹⁰

During the last few years, different compounds against several forms of leishmaniasis have been isolated from natural sources or have been designed, synthesized and tested, - *i.e.* the

anticancer tamoxifen,¹¹ chalcones,¹² bisphosphonates,¹³ and polyamines¹⁴ and also many pentamidine analogs.¹⁵

Tubulin is a globular protein that assembles into microtubules *in vivo* and *in vitro* under the proper conditions. Microtubules play critical roles in mitosis, the maintenance of cell shape, cell motility, and organelle transport in eukaryotes. It has been postulated that different compounds have tubulin as a target, including drugs to treat cancer, fungal and helminthal infections. Among those compounds are anthelmintic benzimidazols, like oxfendazole and thiabendazole, and also some herbicidal dinitroanilines such as trifluralin and oryzalin. All of these have shown to possess a phylogenetic selectivity for tubulin. Werbovetz and collaborators, based on those precedents, have proposed and validated that tubulin can also be a target to design new antileishmanial agents.¹⁶ Over the last ten years an extensive work has been conducted preparing different libraries of antitubulin herbicidal analogs generating a group of promising drug candidates for antileishmanial chemotherapy.¹⁷

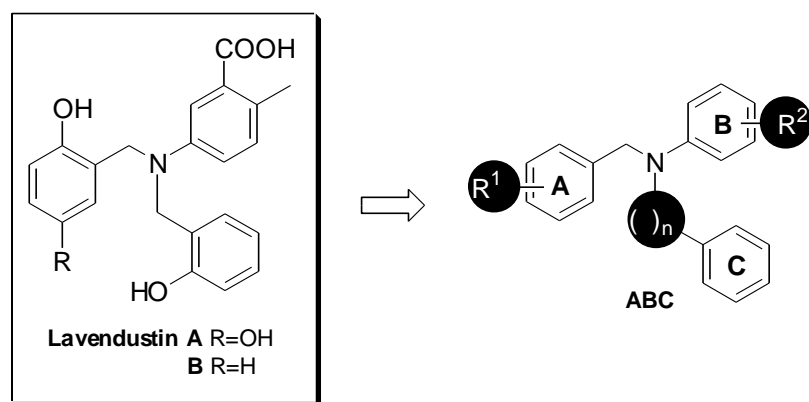


Figure 2. Lavendustins **A** and **B**, and general structure of the designed analogs.

Lavendustin A is a natural metabolite that was first isolated by Onoda from *Streptomyces griseolavendus* in 1989. The initial reported activity of Lavendustin was protein-tyrosine kinase inhibition, but later this compound and its synthetic partial structure have been validated as tubulin polymerization inhibitors.¹⁸

As previously stated, tubulin is a validated target for the design of antileishmanial drugs and lavendustin is an inhibitor of tubulin polymerization, we envisioned that this natural product would be a privileged scaffold to design new synthetic analogs with potential activity against leishmaniasis.

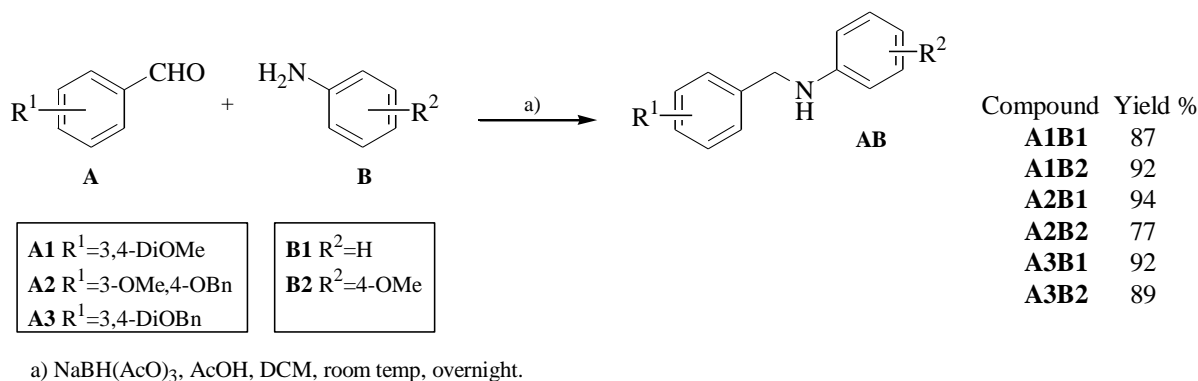
Based on the structure of lavendustin, a series of analogs **ABC** were proposed (Figure 2). The analogs preserve the benzyl and aniline moieties, respectively named rings **A** and **B**. We chose to introduce diversity through substitutions on aromatic rings **A** and **B** and the number of methylenes groups between ring **C** and the central nitrogen.

As part of our program to design and synthesize new agents against parasitic diseases, we describe herein the synthesis of a series of 12 *N,N*-disubstituted anilines and their activities against *Leishmania donovani* promastigotes.

Synthesis

Based on the general **ABC** structure, a series of 12 compounds was prepared through 6 common intermediates. A concise synthetic strategy was designed that starts with a reductive amination of the benzaldehydes and the aniline completing the synthesis with the *N*-alkylation of the secondary amine intermediate. The substituents chosen for rings A and B were benzyloxy and methoxyl groups, in order to prevent oxidative degradation and to simplify the synthesis.

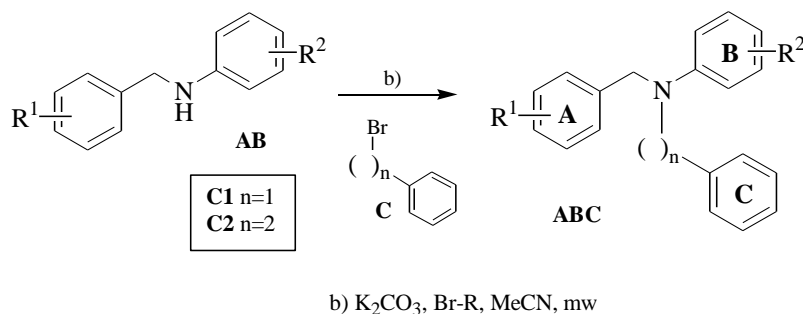
Commercial benzaldehydes and anilines were mixed with NaBH(OAc)₃ and AcOH in dichloromethane¹⁹ to produce 6 different substituted anilines. After purification, the necessary intermediates were obtained in 89 % average yield (Scheme 1).



Scheme 1. Reductive amination of substituted aldehydes **A**.

The last step involved an *N*-alkylation of the secondary amines **AB**. This reaction usually works efficiently with several soft nucleophiles. On the other hand, for some nitrogen-containing nucleophiles like aniline derivatives, the benzylation can be extremely slow (1–4 days) even at an elevated temperature. Initially, standard conditions were used, where the amines **AB** were treated with benzyl bromide in DMF using potassium carbonate as base at 100 °C for 18 hours. Under those conditions, it was observed that reaction mixture contained degradation products, while obtaining the expected tertiary amine **ABC** after purification only in moderate yields. To improve the yield and decrease reaction time, a microwave accelerated reaction was tested. This reaction was initially conducted under microwave irradiation using the same solvent and base that were used under normal heating, producing the expected acceleration of the reaction, decreasing the reaction time from 18 hours to 10-30 minutes, depending of the substrate. After that, the solvent was also studied considering the microwave energy to heat transferability according to the loss angle ($\tan \delta$).²⁰ Reactions using different solvents were tested (DMF, THF and toluene), being acetonitrile the one which performed better. With these conditions in hand,

the 6 *N*-substituted anilines were alkylated using benzyl bromide and 2-bromoethyl benzene (phenethyl bromide), using potassium carbonate as base in acetonitrile. The purified 12 lavendustin analogs were obtained with an 85 % average yield. (Table 1).



Scheme 2. N-Alkylation of secondary amine intermediates **AB**.

Biological activity

The lavendustin analogs **ABC** were tested *in vitro* against *Leishmania donovani* promastigotes. The activities of the compounds are shown on Table 1. As shown, most of the compounds were active with the exception of **A1B1C2** and **A3B1C1** (the maximum concentration tested was 40 µg/mL). The range of activity for the remaining ten compounds was narrow, being 6.5 µg/mL the most active, and 40 µg/mL the less active. The activity (determined as IC₅₀) of the most active compound **A1B1C1** was 6.5 µg/mL when for the control antileishmanial drugs pentamidine was and 1.7 µg/mL, and for Amphotericin B was 0.15 µg/mL.

Table 1. Antileishmanial activity of ABC products

Compound	n	N-benylation <i>L. donovani</i>	
		Yield %	IC ₅₀ µg/mL
A1B1C1	1	91	6.5
A1B1C2	2	87	NA
A1B2C1	1	90	20
A1B2C2	2	91	26
A2B1C1	1	81	26
A2B1C2	2	88	20
A2B2C1	1	84	16
A2B2C2	2	86	26
A3B1C1	1	83	NA
A3B1C2	2	87	40
A3B2C1	1	78	35
A3B2C2	2	77	26
Amphotericin B			0.15
Pentamidine			1.7

NA= non active.

Since antitubulin compounds have also shown promising activity against different apicomplexan parasites including the causative agent of malaria,²¹ we decided to test the antimalarial activity of the compounds prepared against two different strains of *Plasmodium falciparum*, one sensitive and another resistant to chloroquine. (D6, Sierra Leone, sensitive; W2, IndoChina, resistant). Unfortunately none of the compounds showed activity below 4.75 $\mu\text{g/mL}$, a value considered inactive compared to the control drugs artemisinin (IC_{50} =5.3 ng/mL for D6, IC_{50} =4.9 ng/mL for W2) and chloroquine (IC_{50} =9.5 ng/mL for D6, IC_{50} =148,5 ng/mL for W2). The cytotoxicity test toward Vero cells revealed that all the compounds were non-cytotoxic at 4.75 $\mu\text{g/mL}$.

A more detailed analysis of the activity of these compounds can be done by grouping into families according to the building block **C**. (Table 2) In the **C1** family, when the **B1** derivatives are analyzed, it is clear that increasing the size of the phenol substituents decreased the activity. In the **B2** group, this behavior is not observed, having a similar IC_{50} on average. Moving to the **C2** family, similar structural-activity was observed, with activities for **B1** analogs that decrease from **A1** to **A3** with **B2** analogs with same IC_{50} values no matter the phenol substituent. The only exception is **A1B1C2** that was surprisingly inactive. In general terms, the introduction of a second methylene on the **C** building block had no effect on the antileishmanial activity, not producing any substantial change on the activities besides the effect on **A1B1**. In summary, the best activity was observed with dimethoxy substitution on the **A** ring, no oxygenated substituent on the **B** ring and N-Benzyl group for the ring **C**.

Table 2. Antileishmanial activity* ordered by families

C1		B1	B2	C2		B1	B2
R¹\R²		H	OMe	R¹\R²		H	OMe
A1	DiMeO	6.5	20	A1	DiMeO	NA	26
A2	MeO,BnO	20	16	A2	MeO,BnO	20	26
A3	DiBnO	NA	35	A3	DiBnO	40	26

*The values given are IC_{50} ($\mu\text{g/ml}$).

Conclusions

A straightforward synthetic route was used to prepare a small collection of compounds introducing a microwave accelerated *N*-alkylation in very good yields. The antileishmanial activity of this family of 12 lavendustin analogs clearly validated this natural product as a valuable scaffold to prepare new antileishmanial drugs. Further studies will include the preparation of new collections of analogs with halogenated and non-polar substituents on the **B** ring and different patterns of dimethoxy substitution on the **A** ring.

Experimental Section

General. ^1H and ^{13}C NMR spectra were measured on a 400 MHz Bruker Avance DRX or a 300 MHz Bruker Avance II using CDCl_3 as solvent. Chemical shifts were reported in ppm downfield from tetramethylsilane (δ) as the internal standard and coupling constants are in hertz (Hz). Assignments of proton resonances were confirmed by correlated spectroscopy (HH COSY and Heteronuclear Single Quantum Coherence, HSQC) high-resolution mass spectra (HRMS) were recorded on a Micromass spectrometer with lock spray source or Bruker MicroTOF II. Microwave accelerated reactions were performed in a CEM discovery microwave reactor. All the melting points were determined in open Pyrex capillaries with a Electrothermal 9000 melting point apparatus and are uncorrected. The reaction progress was monitored on pre-coated silica gel G or GP TLC plates. Spots were visualized under 254 nm UV light and/or by dipping the TLC plate into a solution of 2 mL anisaldehyde and 10 mL glacial acetic acid and 5 mL H_2SO_4 in 340 mL MeOH followed by heating with a heat gun. Column chromatography was performed with silica gel 60 (230-400 mesh) under a low pressure of nitrogen, using increasing EtOAc-hexane gradients as solvent. All the solvents (hexane, ethyl acetate, CH_2Cl_2 , Et_2O) were distilled prior to use. All reactions were performed under an atmosphere of nitrogen or argon using oven-dried glassware and standard syringe/septa techniques. CH_2Cl_2 was dried over P_2O_5 . DMF was distilled from BaO.

General procedure for reductive amination

To a solution of aldehyde (1.0 equiv) in 10 mL of dichloromethane, amine (1.1 equiv), $\text{NaBH}(\text{AcO})_3$ (1.4 equiv) and finally acetic acid (1.1 equiv) were added in this order and the reaction mixture was stirred overnight at room temperature. Then, the reaction was quenched by addition of 5 % NaHCO_3 (30 mL) and the layers were separated and the aqueous phase was extracted with ether (4 x 10 mL). Combined organic extracts were dried over Na_2SO_4 and concentrated. The products were purified by column chromatography.

***N*-(3,4-Dimethoxybenzyl)aniline (A1B1).** 3,4-dimethoxybenzaldehyde (104 mg, 0.63 mmol); aniline (63 μL , 0.69 mmol) in dry dichloromethane (6 mL); sodium triacetoxyborohydride (204 mg, 0.96 mmol) and acetic acid (64 μL). Purification by column chromatography provides 133 mg (87 %) of the amine **A1B1** as yellowish oil. ^1H NMR (400 MHz CDCl_3): 7.25-7.21 (m, 2H), 6.97-6.68 (m, 6H), 4.29 (s, 2H, $\text{C}_{\text{A1}}\text{-CH}_2$), 3.91 (s, 3H, $\text{C}_{\text{A4}}\text{-OMe}$), 3.90 (s, 3H, $\text{C}_{\text{A3}}\text{-OMe}$). ^{13}C NMR (100 MHz CDCl_3): 149.3 (C_{B1} , C), 148.3 ($\text{C}_{\text{A3}}\text{-C}_{\text{A4}}$, C), 132.1 (C_{A1} , C), 129.3 ($\text{C}_{\text{B2}}\text{-C}_{\text{B6}}$, C), 119.7 (C_{A6} , CH), 117.6 (C_{B4} , CH), 112.9 ($\text{C}_{\text{B2}}\text{-C}_{\text{B6}}$, CH), 111.4 (C_{A5} , CH), 110.9 (C_{A2} , CH), 56.0 and 55.9 ($\text{C}_{\text{A3}}\text{-OMe}$, $\text{C}_{\text{A4}}\text{-OMe}$), 48.3 ($\text{C}_{\text{A1}}\text{-CH}_2$). ESI-HRMS Calcd for ($\text{M}+\text{Na}^+$) $\text{C}_{15}\text{H}_{17}\text{NO}_2\text{Na}$: 266.1157, found: 266.1147.

***N*-(3,4-Dimethoxybenzyl)-4-methoxyaniline (A1B2).** 3,4-dimethoxybenzaldehyde (110 mg, 0.66 mmol); p-anisidine (90 mg, 0.73 mmol) in dry dichloromethane (6.5 mL); sodium triacetoxyborohydride (216 mg, 1.02 mmol) and acetic acid (67 μL). Purification by column chromatography provides 166 mg (92 %) of the amine **A1B2** as white crystalline solid. Mp 114-

116 °C. ^1H NMR (400 MHz CDCl_3): 6.95-6.80 (m, 4H, aromatic), 6.65-6.63 (m, 3H, , aromatic), 4.23 (s, 2H, $\text{C}_{\text{A}1}\text{-CH}_2$), 3.89 (s, 3H, $\text{C}_{\text{A}3}\text{-OMe}$), 3.88 (s, 3H, $\text{C}_{\text{A}4}\text{-OMe}$), 3.76 (s, 3H $\text{C}_{\text{B}4}\text{-OMe}$). ^{13}C NMR (100 MHz CDCl_3): 152.1 ($\text{C}_{\text{B}4}$, C), 149.0 ($\text{C}_{\text{A}3}$, C), 148.1 ($\text{C}_{\text{A}4}$, C), 142.4 ($\text{C}_{\text{B}1}$, C), 132.2 ($\text{C}_{\text{A}1}$, C), 119.5($\text{C}_{\text{A}6}$, CH), 114.8 ($\text{C}_{\text{B}2}\text{-C}_{\text{B}6}$, CH), 114.0 ($\text{C}_{\text{B}3}\text{-C}_{\text{B}5}$, CH), 111.2 ($\text{C}_{\text{A}2}$, CH), 110.8 ($\text{C}_{\text{A}5}$, CH), 55.8 ($\text{C}_{\text{B}4}\text{-OMe}$), 55.7 ($\text{C}_{\text{A}4}\text{-OMe}$), 55.6 ($\text{C}_{\text{A}3}\text{-OMe}$), 49.0 ($\text{C}_{\text{A}1}\text{-CH}_2$). ESI-HRMS Calcd for ($\text{M}+\text{Na}^+$) $\text{C}_{16}\text{H}_{19}\text{NO}_3\text{Na}$: 296.1250, found: 296.1263.

***N*-(4-(Benzyloxy)-3-methoxybenzyl)aniline (A2B1)**. 4-benzyloxy-3-methoxybenzaldehyde (108 mg, 0.45 mmol); aniline (45 μL , 0.49 mmol) in dry dichloromethane (4 mL); sodium triacetoxyborohydride (145 mg, 0.69 mmol) and acetic acid (45 μL). Purification by column chromatography provides 134 mg (94 %) of the amine **A2B1** as a yellow solid. Mp 76-77 °C. ^1H NMR (400 MHz CDCl_3): 7.53-7.25 (m, 7H, aromatic), 7.04-6.69 (m, 6H, aromatic), 5.20 (s, 2H, $\text{C}_{\text{A}4}\text{-OCH}_2\text{Ph}$), 4.27 (s, 2H, $\text{C}_{\text{A}4}\text{-OCH}_2$), 3.95 (s, 3H, $\text{C}_{\text{A}3}\text{-OMe}$). ^{13}C NMR (100 MHz CDCl_3): 148.8 ($\text{C}_{\text{B}1}$,C), 148.2 ($\text{C}_{\text{A}4}$,C), 148.1 ($\text{C}_{\text{A}3}$, C), 136.9 ($\text{C}_{\text{A}1}$, C), 131.9 (OCH_2Ph), 129.1 (OCH_2Ph), 128.4 (OCH_2Ph), 127.7 (OCH_2Ph), 127.3 ($\text{C}_{\text{B}3}\text{-C}_{\text{B}5}$, CH), 120.2 ($\text{C}_{\text{A}6}$, CH), 117.4 ($\text{C}_{\text{B}4}$, CH), 113.6 ($\text{C}_{\text{A}2}$, CH), 112.7 ($\text{C}_{\text{B}2}\text{-C}_{\text{B}6}$, CH), 111.9 ($\text{C}_{\text{A}5}$, CH), 70.9 ($\text{C}_{\text{A}4}\text{-OCH}_2\text{Ph}$), 55.9 ($\text{C}_{\text{A}3}\text{-OCH}_3$), 47.8($\text{C}_{\text{A}1}\text{-CH}_2$). ESI-HRMS Calcd for ($\text{M}+\text{Na}^+$) $\text{C}_{21}\text{H}_{21}\text{NO}_2\text{Na}$: 342.1470, found: 342.1472.

***N*-(4-(Benzyloxy)-3-methoxybenzyl)-4-methoxyaniline (A2B2)**. 4-benzyloxy-3-methoxybenzaldehyde (110 mg, 0.45 mmol); p-anisidine (62 mg, 0.5 mmol) in dry dichloromethane (5 mL); sodium triacetoxyborohydride (148 mg, 0.7 mmol) and acetic acid (45 μL). Purification by column chromatography provides 122 mg (77 %) of the amine **A2B2** as a yellow solid. Mp 103-104 °C. ^1H NMR (400 MHz CDCl_3): 7.48-7.34 (m, 5H, aromatic), 7.00-6.61 (m, 7H, aromatic), 5.16 (s, 2H, OCH_2Ph), 4.19 (s, 2H, $\text{C}_{\text{A}3}\text{-OCH}_3$), 3.91 (s, 3H, $\text{C}_{\text{A}4}\text{-OCH}_3$), 3.78 (s, 3H $\text{C}_{\text{B}4}\text{-OMe}$), 3.54 (bs, 1H, NH). ^{13}C NMR (100 MHz CDCl_3): 152.1 ($\text{C}_{\text{B}4}$, C), 148.8 ($\text{C}_{\text{A}4}$, C), 148.2 ($\text{C}_{\text{A}3}$, C), 142.4 ($\text{C}_{\text{B}1}$, C), 137.0 (OCH_2Ph), 132.2 (OCH_2Ph), 128.4 (OCH_2Ph), 127.7 (OCH_2Ph), 127.3 ($\text{C}_{\text{A}6}$, CH), 120.2 ($\text{C}_{\text{B}2}\text{-C}_{\text{B}6}$, CH), 116.3 ($\text{C}_{\text{B}3}\text{-C}_{\text{B}5}$, CH), 114.8 ($\text{C}_{\text{A}2}$, CH), 114.0 ($\text{C}_{\text{A}5}$, CH), 70.9 (OCH_2Ph), 55.9 ($\text{C}_{\text{B}4}\text{-OMe}$), 55.7($\text{C}_{\text{A}3}\text{-OMe}$), 48.8 ($\text{C}_{\text{A}1}\text{-CH}_2$). ESI-HRMS Calcd for ($\text{M}+\text{Na}^+$) $\text{C}_{22}\text{H}_{23}\text{NO}_3\text{Na}$: 372.1576, found: 372.1564.

***N*-(3,4-Bis(benzyloxy)benzyl)aniline (A3B1)**. 3,4-bis(benzyloxy)benzaldehyde (106 mg, 0.33 mmol); aniline (44 μL , 0.37 mmol) in dry dichloromethane (3 mL); sodium triacetoxyborohydride (109 mg, 0.51 mmol) and acetic acid (37 μL). Purification by column chromatography provides 121 mg (92 %) of the amine **A3B1** as a white solid. Mp 126-127 °C. ^1H NMR (400 MHz CDCl_3): 7.59-7.29 (m, 13H, aromatic), 7.12-6.72 (m, 5H, aromatic), 5.26 (s, 2H, $\text{C}_{\text{A}4}\text{-OCH}_2\text{Ph}$), 5.25 (s, 2H, $\text{C}_{\text{A}3}\text{-OCH}_2\text{Ph}$), 4.30 (s, 2H, $\text{C}_{\text{A}1}\text{-CH}_2$), 3.93 (s, 1H, NH). ^{13}C NMR (100 MHz CDCl_3): 149.3 ($\text{C}_{\text{B}1}$, C), 148.4 ($\text{C}_{\text{A}3}\text{-C}_{\text{A}4}$, C), 137.6 (OCH_2Ph), 133.1 ($\text{C}_{\text{A}1}$, CH), 129.4 (OCH_2Ph), 128.6 (OCH_2Ph), 127.6 (OCH_2Ph), 127.5 ($\text{C}_{\text{B}3}\text{-C}_{\text{B}5}$, CH), 120.6 ($\text{C}_{\text{A}6}$, CH), 117.7 ($\text{C}_{\text{B}4}$, CH), 115.2 ($\text{C}_{\text{A}5}$, CH), 114.7 ($\text{C}_{\text{A}2}$, CH), 113.1 ($\text{C}_{\text{B}2}\text{-C}_{\text{B}6}$, CH), 71.6 ($\text{C}_{\text{A}3}\text{-OCH}_2\text{Ph}$), 71.4($\text{C}_{\text{A}4}\text{-OCH}_2\text{Ph}$), 48.1 ($\text{C}_{\text{A}1}\text{-CH}_2$). ESI-HRMS ($\text{M}+\text{Na}^+$) $\text{C}_{27}\text{H}_{25}\text{NO}_2\text{Na}$: 418,1783 found: 418.1771.

***N*-(3,4-Bis(benzyloxy)benzyl)-4-methoxyaniline (A3B2)**. 3,4-bis(benzyloxy)benzaldehyde (110 mg, 0.35 mmol); p-anisidine (47 mg, 0.38 mmol) in dry dichloromethane (3.5 mL); sodium

triacetoxyborohydride (113 mg, 0.53 mmol) and acetic acid (35 μ L). Purification by column chromatography provides 131 mg (89 %) of the amine **A3B2** as a yellow solid. Mp 132-133 $^{\circ}$ C. ^1H NMR (400 MHz CDCl_3): 7.52-7.36 (m, 10H, aromatic), 7.06-6.62 (m, 7H, aromatic), 5.20 (s, 2H, $\text{C}_{\text{A}3}\text{-OCH}_2\text{Ph}$), 5.19 (s, 2H, $\text{C}_{\text{A}4}\text{-OCH}_2\text{Ph}$), 4.21 (s, 2H, $\text{C}_{\text{A}3}\text{-OCH}_2\text{Ph}$), 3.79 (s, 3H, $\text{C}_{\text{B}4}\text{-OMe}$). ^{13}C NMR (100 MHz CDCl_3): 152.1 ($\text{C}_{\text{B}4}$, C), 149.1 ($\text{C}_{\text{A}3}$, C), 148.1 ($\text{C}_{\text{A}4}$, C), 142.4 ($\text{C}_{\text{B}1}$, C), 137.2 (OCH_2Ph), 133.1 (OCH_2Ph), 128.4 (OCH_2Ph), 127.7 (OCH_2Ph), 127.2 (OCH_2Ph), 120.4 ($\text{C}_{\text{A}6}$, CH), 115.3 ($\text{C}_{\text{B}2}\text{-C}_{\text{B}6}$, CH), 114.9 ($\text{C}_{\text{B}3}\text{-C}_{\text{B}5}$, CH), 114.6 ($\text{C}_{\text{A}5}$, CH), 114.1 ($\text{C}_{\text{A}2}$, CH), 71.4 ($\text{C}_{\text{A}4}\text{-OCH}_2\text{Ph}$), 71.2 ($\text{C}_{\text{A}3}\text{-OCH}_2\text{Ph}$), 55.7 ($\text{C}_{\text{B}4}\text{-OMe}$), 48.9 ($\text{C}_{\text{A}1}\text{-CH}_2$). ESI-HRMS Calcd for ($\text{M}+\text{Na}^+$) $\text{C}_{28}\text{H}_{27}\text{NO}_3\text{Na}$: 448.1889, found: 448.1908.

General procedure for microwave *N*-alkylation

To a solution of amine (1 eq.) in dry acetonitrile prepared inside the microwave reaction tube, anh. K_2CO_3 (1.3 eq.) and benzyl bromide (1.3 eq.) were added. The sealed tube was heated on the microwave reactor using the microwave settings describe below. The reaction mixture was poured into 30 mL of brine extracted with dichloromethane (4 x 10 mL). Combined organic extracts were dried over magnesium sulfate and evaporated. The compound was purified by flash column chromatography.

Microwave settings:

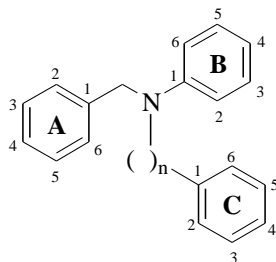
Temp = 150 $^{\circ}$ C, Power: 100 W, Run time= 10 min, Hold time = 20 min.

Table 3. Microwave *N*-alkylation of AB secondary amines

Secondary amine	Acetonitrile	K_2CO_3	R-Br	Isolated product
A1B1 56 mg, 0.23 mmol	3 mL	54 mg, 0.39 mmol	BnBr 36 μ L, 0.30 mmol	A1B1C1 70 mg (91 %)
A1B1 61 mg, 0.25 mmol	3 mL	58 mg, 0.42 mmol	PhCH ₂ CH ₂ Br 45 μ L, 0.33 mmol	A1B1C2 76 mg (87 %)
A1B2 60 mg, 0.22 mmol	3 mL	51 mg, 0.37 mmol	BnBr 45 μ L, 0.33 mmol	A1B2C1 72 mg (90 %)
A1B2 60 mg, 0.22 mmol	3 mL	51 mg, 0.37 mmol	PhCH ₂ CH ₂ Br 39 μ L, 0.29 mmol	A1B2C2 75 mg (91 %)
A2B1 56 mg, 0.18 mmol	3 mL	41 mg, 0.30 mmol	BnBr 27 μ L, 0.23 mmol	A2B1C1 58 mg (81 %)
A2B1 61 mg, 0.19 mmol	3 mL	45 mg, 0.32 mmol	PhCH ₂ CH ₂ Br 34 μ L, 0.25 mmol	A2B1C2 71 mg (88 %)
A2B2 68 mg, 0.19 mmol	3 mL	45 mg, 0.33 mmol	BnBr 30 μ L, 0.25 mmol	A2B2C1 72 mg (84 %)
A2B2 70 mg, 0.18 mmol	3 mL	41 mg, 0.30 mmol	PhCH ₂ CH ₂ Br 32 μ L, 0.23 mmol	A2B2C2 69 mg (86 %)

Table 3. (Continued)

A3B1		41 mg, 0.30	BnBr	A3B1C1
70 mg, 0.18 mmol	3 mL	mmol	27 μ L, 0.23 mmol	60 mg (83 %)
A3B1		44 mg, 0.32	PhCH ₂ CH ₂ Br	A3B1C2
74 mg, 0.19 mmol	3 mL	mmol	33 μ L, 0.24 mmol	69 mg (87 %)
A3B2		31 mg, 0.23	BnBr	A3B2C1
53 mg, 0.13 mmol	3 mL	mmol	21 μ L, 0.17 mmol	46 mg (78 %)
A3B2		31 mg, 0.22	PhCH ₂ CH ₂ Br	A3B2C2
52 mg, 0.13 mmol	3 mL	mmol	23 μ L, 0.17 mmol	46 mg (77 %)



Numbering by assignment on NMR-spectra

***N*-Benzyl-*N*-(3,4-dimethoxybenzyl)aniline (A1B1C1)**. Yellowish oil. ¹H NMR (300 MHz CDCl₃): 7.37-7.19 (m, 7H, aromatic), 6.83-6.72 (m, 5H, aromatic), 4.65 (s, 2H, C_{A1}-CH₂), 4.61 (s, 2H, C_{C1}-CH₂), 3.89 (s, 3H, C_{A3}-OMe), 3.82 (s, 3H, C_{A4}-OMe). ¹³C NMR (75 MHz CDCl₃): 149.4 (C_{A3}, C), 149.3 (C_{B1}, C), 148.0 (C_{A4}, C), 138.7 (C_{C1}, C), 131.1 (C_{B6}, CH), 129.2 (C_{B3}-C_{B5}, CH), 128.6 (C_{C3}-C_{C5}, CH), 126.9 (C_{C2}-C_{C6}, CH), 126.8 (C_{C4}, CH), 118.8 (C_{A6}, CH), 116.9 (C_{A4}, C), 112.7 (C_{B2}-C_{B6}, CH), 111.3 (C_{A2}, CH), 110.0 (C_{A5}, C), 56.0 (C_{A3}-OMe), 55.9 (C_{A4}-OMe), 54.2 (C_{A1}-CH₂), 54.1 (C_{C1}-CH₂). ESI-HRMS Calcd for (M+Na⁺) C₂₂H₂₃NO₂Na: 356.1626, found: 356.1641.

***N*-(3,4-Dimethoxybenzyl)-*N*-phenethylaniline (A1B1C2)**. White solid. Mp 92-94 °C. ¹H NMR (300 MHz CDCl₃): 7.34-7.21 (m, 8H, aromatic), 6.81-6.74 (m, 5H, aromatic), 4.45 (s, 2H, C_{A1}-CH₂), 3.88 (s, 3H, C_{A3}-OMe), 3.83 (s, 3H, C_{A4}-OMe), 3.65 (dd, 2H, *J* = 7.56 and 8.05, C_{C1}-CH₂CH₂Ph), 2.95 (dd, 2H, *J* = 8.06 and 7.55, C_{C1}-CH₂CH₂Ph). ¹³C NMR (75 MHz CDCl₃): 149.2 (C_{A3}, C), 148.4 (C_{A4}, C), 147.9 (C_{B1}, C), 139.6 (C_{C1}, C), 131.3 (C_{A1}, CH), 129.3 (C_{B3}-C_{B5}, CH), 128.8 (C_{C2}-C_{C6}, CH), 128.6 (C_{C3}-C_{C5}, CH), 126.3 (C_{C4}, CH), 118.7 (C_{A6}, CH), 116.5 (C_{B4}, CH), 112.5 (C_{B2}-C_{B6}, CH), 111.3 (C_{A5}, CH), 109.8 (C_{A2}, C), 56.0 (C_{A4}-OMe), 55.9 (C_{A3}-OMe), 54.3 (C_{A1}-CH₂), 52.8 (C_{C1}-CH₂CH₂Ph), 33.4 (C_{C1}-CH₂CH₂Ph). ESI-HRMS Calcd for (M+Na⁺) C₂₃H₂₅NO₂Na: 370.1778, found: 370.1772.

***N*-Benzyl-*N*-(3,4-dimethoxybenzyl)-4-methoxyaniline (A1B2C1)**. Yellow oil. ¹H NMR (300 MHz CDCl₃): 7.41-7.27 (m, 5H, aromatic), 6.85-6.74 (m, 7H, aromatic), 4.55 (s, 2H, C_{A1}-CH₂), 4.51 (s, 2H, C_{C1}-CH₂), 3.89 (s, 3H, C_{A3}-OMe), 3.82 (s, 3H, C_{A4}-OMe), 3.76 (s, 3H, C_{B4}-OMe). LC-ESI-MS (M+H⁺): 363.11.

***N*-(3,4-Dimethoxybenzyl)-4-methoxy-*N*-phenethylaniline (A1B2C2).** Yellow oil.

¹H NMR (300 MHz CDCl₃): 7.34-7.20 (m, 5H, aromatic), 6.89-6.79 (m, 7H, aromatic), 4.38 (s, 2H, C_{A1}-CH₂), 3.89 (s, 3H C_{A4}-OMe), 3.84 (s, 3H, C_{A3}-OMe), 3.79 (s, 3H, C_{B4}-OMe), 3.58 (dd, 2H, *J* = 8.06 and 7.55, C_{C1}-CH₂CH₂Ph), 2.92 (dd, 2H, *J* = 8.05 and 7.06, C_{C1}-CH₂CH₂Ph). ¹³C NMR (75 MHz CDCl₃): 151.8 (C_{B4}, C), 149.1 (C_{A3}, C), 147.9 (C_{A4}, C), 143.2 (C_{A1}, C), 139.9 (C_{C1}, C), 131.7 (C_{A1}, C), 128.8 (C_{C2}-C_{C6}, CH), 128.5 (C_{C3}-C_{C5}, CH), 126.2 (C_{C4}, CH), 119.0 (C_{A6}, CH), 115.0 (C_{B2}-C_{B6}, CH), 114.9 (C_{B3}-C_{B5}, CH), 111.2 (C_{A5}, CH), 110.2 (C_{A2}, CH), 55.9 (C_{B4}-OMe), 55.8 (C_{A4}-OMe), 55.8 (C_{A3}-OMe), 55.5 (C_{A1}-CH₂), 53.5 (C_{C1}-CH₂CH₂Ph), 33.4 (C_{C1}-CH₂CH₂Ph). ESI-HRMS Calcd for (M+H⁺) C₂₄H₂₈NO₃: 378.2069, found: 378.2087.

***N*-Benzyl-*N*-(4-(benzyloxy)-3-methoxybenzyl)aniline (A2B1C1).** White solid. Mp 105-106 °C.

¹H NMR (300 MHz CDCl₃): 7.37-7.19 (m, 12H, aromatic), 6.86-6.74 (m, 6H, aromatic), 5.10 (s, 2H, OCH₂Ph), 4.55 (s, 2H, C_{A1}-CH₂), 4.53 (s, 2H, C_{C1}-CH₂), 3.91 (s, 3H, C_{A3}-OMe). ¹³C NMR (75 MHz CDCl₃): ¹³C NMR (75 MHz CDCl₃): 149.5 (C_{A4}-C_{B1}, C), 147.5 (C_{A3}, C), 138.8 (C_{C1}, C), 137.3 (C_{A1}, C), 134.5 (OCH₂Ph), 129.4 (C_{B3}-C_{B5}, CH), 128.6 (C_{C3}-C_{C5}, CH), 128.5 (OCH₂Ph), 127.7 (OCH₂Ph), 127.5 (C_{C4}, CH), 126.9 (C_{B4}, CH), 126.7 (C_{C2}-C_{C6}, CH), 121.4 (C_{A6}, CH), 115.3 (C_{A2}, CH), 112.6 (C_{B2}-C_{B6}, CH), 111.9 (C_{A5}, CH), 71.0 (OCH₂Ph), 56.0 (C_{A3}-OMe), 54.4 (C_{A1}-CH₂), 54.3 (C_{C1}-CH₂). ESI-HRMS Calcd for (M+Na⁺) C₂₈H₂₈NO₂Na: 432.1934, found: 432.1923.

***N*-(4-(Benzyloxy)-3-methoxybenzyl)-*N*-phenethylaniline (A2B1C2).** White solid. Mp 77.5-78.5 °C. ¹H NMR (400 MHz CDCl₃): 7.37-7.18 (m, 14H, aromatic), 6.85-6.74 (m, 4H, aromatic), 5.09 (s, 2H, OCH₂Ph), 4.38 (s, 2H C_{A1}-CH₂), 3.89 (s, 3H, C_{A4}-OMe), 3.60 (dd, 2H, *J* = 8.06 and 7.3, C_{C1}-CH₂CH₂Ph), 3.19 (dd, 2H, *J* = 7.81 and 7.55, C_{C1}-CH₂CH₂Ph).

¹³C NMR (100 MHz CDCl₃): 148.6 (C_{A4}, C), 148.3 (C_{A3}, C), 139.6 (C_{B1}, C), 137.1 (C_{C1}, C), 131.3 (C_{A1}, C), 129.3 (OCH₂Ph), 128.8 (C_{C3}-C_{C5}, CH), 128.5 (C_{C2}-C_{C6}, CH), 127.8 (C_{C3}-C_{C5}, CH), 127.4 (OCH₂Ph), 126.2 (C_{C4}, CH), 119.3 (C_{A6}, CH), 116.4 (C_{B4}, CH), 112.8 (C_{B3}-C_{B5}, CH), 112.3 (C_{B2}-C_{B6}, CH), 111.9 (C_{A5}, CH), 71.0 (OCH₂Ph), 56.1 (C_{A3}-OMe), 54.0 (C_{A1}-CH₂), 52.7 (C_{C1}-CH₂CH₂Ph), 33.4 (C_{C1}-CH₂CH₂Ph)

LC-ESI-MS (M+H⁺): 424.13.

***N*-Benzyl-*N*-(4-(benzyloxy)-3-methoxybenzyl)-4-methoxyaniline (A2B2C1).** Colorless oil.

¹H NMR (300 MHz CDCl₃): 7.39-7.22 (m, 10H, aromatic), 6.87-6.68 (m, 7H, aromatic), 5.09 (s, 2H, OCH₂Ph), 4.44 (s, 4H, C_{A1}-CH₂, C_{C1}-CH₂), 3.90 (s, 3H, C_{B4}-OMe), 3.77 (s, 3H, C_{B4}-OMe). ¹³C NMR (75 MHz CDCl₃): 151.8 (C_{B4}, C), 148.5 (C_{A4}, C), 148.2 (C_{A3}, C), 143.9 (C_{B1}, C), 139.1 (C_{C1}, C), 137.2 (C_{A1}, C), 131.3 (OCH₂Ph), 128.5 (OCH₂Ph), 128.1 (OCH₂Ph), 127.7 (OCH₂Ph), 127.3 (OCH₂Ph), 127.0 (C_{C6}-C_{C2}, CH), 126.8 (C_{C4}, CH), 119.6 (C_{A6}, CH), 114.8 (C_{B3}-C_{B5}, CH), 114.7 (C_{B2}-C_{B6}, CH), 113.1 (C_{A5}, CH), 111.8 (C_{A2}, CH), 70.9 (OCH₂Ph), 56.1 (C_{A3}-OMe), 55.7 (C_{B4}-OMe), 54.9 (C_{A1}-CH₂), 54.7 (C_{C1}-CH₂). ESI-HRMS Calcd for (M+Na⁺) C₂₉H₂₉NO₃Na: 462.2040, found: 462.2035.

***N*-(4-(Benzyloxy)-3-methoxybenzyl)-4-methoxy-*N*-phenethylaniline (A2B2C2).** Yellow oil.

¹H NMR (400 MHz CDCl₃): 7.44-7.20 (m, 12H, aromatic), 6.90-6.76 (m, 5H, aromatic), 5.13 (s, 2H, OCH₂Ph), 4.35 (s, 2H, C_{A1}-CH₂), 3.92 (s, 3H, C_{A3}-OMe), 3.82 (s, 3H, C_{B4}-OMe), 3.52 (dd,

2H, $J = 7.81$ and 7.80 , $C_{C1}-\underline{CH_2}CH_2Ph$), 2.95 (dd, 2H, $J = 8.06$ and 7.30 , $C_{C1}-CH_2\underline{CH_2}Ph$). ^{13}C NMR (100 MHz $CDCl_3$): 151.8 (C_{B4} , C), 148.7 (C_{A3} , C), 148.3 (C_{A4} , C), 143.2 (C_{B1} , C), 139.9 (C_{C1} , C), 137.3 (OCH_2Ph), 131.8 (C_{A1} , C), 128.8 ($C_{B3}-C_{B5}$, CH), 128.54 ($C_{C3}-C_{C5}$ and OCH_2Ph), 127.8 (OCH_2Ph), 127.4 ($C_{C2}-C_{C6}$ and OCH_2Ph), 126.2 (C_{C4} , CH), 119.7 (C_{A6} , CH), 114.9 ($C_{B2}-C_{B6}$, CH), 113.3 (C_{A5} , CH), 112.0 (C_{A2} , CH), 71.0 (OCH_2Ph), 56.2 ($C_{A3}-OMe$), 55.8 ($C_{B4}-OMe$), 55.3 ($C_{A1}-\underline{CH_2}$), 53.5 ($C_{C1}-\underline{CH_2}CH_2Ph$), 33.5 ($C_{C1}-CH_2\underline{CH_2}Ph$).

ESI-HRMS Calcd for $(M+H^+)$ $C_{30}H_{32}NO_3$: 454.2382 , found: 454.2389 .

***N*-Benzyl-*N*-(3,4-bis(benzyloxy)benzyl)aniline (A3B1C1)**. Yellow oil. 1H NMR (400 MHz $CDCl_3$): $7.48-7.22$ (m, 17H, aromatic), $6.91-6.75$ (m, 6H, aromatic), 5.19 (s, 2H, OCH_2Ph), 5.12 (s, 2H, OCH_2Ph), 4.56 (s, 4H, $C_{A1}-\underline{CH_2}$ and $C_{B1}-\underline{CH_2}$). ESI-HRMS Calcd for $(M+H^+)$ $C_{34}H_{32}NO_2$: 486.2433 , found: 486.2420 .

***N*-(3,4-Bis(benzyloxy)benzyl)-*N*-phenethylaniline (A3B1C2)**. Yellowish oil. 1H NMR (400 MHz $CDCl_3$): $7.47-7.18$ (m, 17H, aromatic), $6.88-6.74$ (m, 6H, aromatic), 5.16 (s, 2H, OCH_2Ph), 5.10 (s, 2H, OCH_2Ph), 4.38 (s, 2H, $C_{A1}-\underline{CH_2}$), 3.55 (dd, 2H, $J = 8.06$ and 7.8 , $C_{C1}-\underline{CH_2}CH_2Ph$), 2.87 (dd, 2H, $J = 8.31$ and 7.31 , $C_{C1}-CH_2\underline{CH_2}Ph$). LC-ESI-MS $(M+H^+)$: 500.22 .

***N*-Benzyl-*N*-(3,4-bis(benzyloxy)benzyl)-4-methoxyaniline (A3B2C1)**. Colorless oil. 1H NMR (300 MHz $CDCl_3$): $7.49-7.25$ (m, 15H, aromatic), $6.91-6.70$ (m, 7H, aromatic), 5.18 (s, 2H, $C_{A4}-OCH_2Ph$), 5.11 (s, 2H, $C_{A3}-OCH_2Ph$), 4.46 (s, 4H, $C_{A1}-\underline{CH_2}$ and $C_{C1}-\underline{CH_2}$), 3.78 (s, 3H, $C_{B4}-OMe$). ^{13}C NMR (75 MHz $CDCl_3$): 151.8 (C_{B4} , C), 149.0 (C_{A3} , C), 147.8 (C_{A4} , C), 143.9 (C_{A1} , C), 137.5 (C_{C1} , C), 137.4 (C_{B1} , C), 132.3 (OCH_2Ph), 128.5 (OCH_2Ph), 128.5 ($C_{B3}-C_{B5}$, CH), 127.8 (OCH_2Ph), 127.8 (OCH_2Ph), 127.4 (OCH_2Ph), 127.3 ($C_{C2}-C_{C6}$, CH), 127.0 ($C_{A3}-OCH_2Ph$), 126.8 (C_{C4} , CH), 119.9 (C_{A6} , CH), 115.2 ($C_{B2}-C_{B6}$, CH), 114.8 ($C_{B3}-C_{B5}$, CH), 114.7 (C_{A2} , CH), 114.0 (C_{A5} , CH), 71.5 ($C_{A4}-OCH_2Ph$), 71.2 ($C_{A3}-OCH_2Ph$), 55.7 ($C_{B4}-OMe$), 55.0 ($C_{A1}-\underline{CH_2}$), 54.8 ($C_{A3}-\underline{CH_2}$). ESI-HRMS Calcd for $(M+H^+)$ $C_{35}H_{34}NO_3$: 516.2533 , found: 516.2519 .

***N*-(3,4-Bis(benzyloxy)benzyl)-4-methoxy-*N*-phenethylaniline (A3B2C2)**. Yellow oil. 1H NMR (400 MHz $CDCl_3$): $7.57-7.26$ (m, 15H, aromatic), $6.97-6.84$ (m, 7H, aromatic), 5.24 (s, 2H, $C_{A3}-OCH_2Ph$), 5.20 (s, 2H, $C_{A4}-OCH_2Ph$), 4.41 (s, 2H, $C_{A1}-\underline{CH_2}$), 3.87 (s, 3H, $C_{B4}-OMe$), 3.59 (dd, 2H, $J = 7.80$ and 7.81 , $C_{C1}-\underline{CH_2}CH_2Ph$), 2.95 (dd, 2H, $J = 8.06$ and 7.05 , $C_{C1}-CH_2\underline{CH_2}Ph$).

^{13}C NMR (75 MHz $CDCl_3$): 151.9 (C_{B4} , C), 149.2 (C_{A3} , C), 148.0 (C_{A4} , C), 143.2 (C_{B1} , C), 139.9 (C_{C1} , C), 137.6 (OCH_2Ph), 137.5 (OCH_2Ph), 132.7 (C_{A1} , C), 128.9 ($C_{C2}-C_{C6}$, CH), 128.5 (OCH_2Ph), 127.8 (OCH_2Ph), 127.4 (OCH_2Ph), 126.3 ($C_{C3}-C_{C5}$, CH), 120.0 (C_{A5} , CH), 119.9 (C_{A6} , CH), 115.4 ($C_{B3}-C_{B5}$, CH), 115.0 ($C_{B2}-C_{B6}$, CH), 114.2 (C_{A2} , CH), 71.6 ($C_{A3}-OCH_2Ph$), 71.3 ($C_{A4}-OCH_2Ph$), 55.8 ($C_{B4}-OMe$), 55.4 ($C_{A1}-\underline{CH_2}$), 53.5 ($C_{C1}-\underline{CH_2}CH_2Ph$), 33.6 ($C_{C1}-CH_2\underline{CH_2}Ph$). LC-ESI-MS $(M+H^+)$: 530.16 .

***In vitro* antileishmanial and antimalarial assays**

Antileishmanial activity of the compounds was tested *in vitro* on a culture of *Leishmania donovani* promastigotes (Strain S1). In a 96 well microplate assay the compounds with

appropriate dilution were added to the promastigotes culture (2×10^6 cell/mL) to get the final concentrations of 40, 8 and 1.6 $\mu\text{g}/\text{mL}$. The plates were incubated at 26 C for 72 hours and growth was determined by Alamar blue assay.²² Pentamidine and Amphotericin B were used as the standard antileishmanial agents. All the analogs were simultaneously tested for cytotoxicity on VERO (monkey kidney fibroblast) cells by Neutral Red assay.²³ IC_{50} value for each compound was computed from the growth inhibition curve. Antimalarial activity was determined *in vitro* on chloroquine sensitive (D6, Sierra Leone) and resistant (W2, IndoChina) strains of *Plasmodium falciparum*. The 96 well microplate assay is based on evaluation of the effect of the compounds on growth of asynchronous cultures of *P. falciparum*, determined by the assay of parasite lactate dehydrogenase (pLDH) activity.²⁴ The appropriate dilutions of the compounds were prepared in DMSO or RPMI-1640 medium and added to the cultures of *P. falciparum* (2% hematocrit, 2% parasitemia) set up in clear flat bottomed 96 well plates. The plates were placed into the humidified chamber and flushed with a gas mixture of 90% N_2 , 5% CO_2 & 5% O_2 . The cultures were incubated at 37C for 48 hours. Growth of the parasite in each well was determined by pLDH assay using Malstat® reagent. The medium and RBC controls were also set-up in each plates. The standard antimalarial agents, chloroquine and artemisinin, were used as the positive controls while DMSO was tested as the negative control.

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