SYNTHESES AND BIOLOGICAL EVALUATION OF (±)-3a-PHENYL CONGENERS OF PHYSOSTIGMINE AND PHENSERINE[1)]

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Abstract- (±)-3a-Phenyl congeners of physostigmine and phenserine, synthesized for the first time by a modification of the Julian total synthesis of physostigmine, were evaluated for anticholinesterase action against human acetyl- and butyrylcholinesterase. These analogues were found to lack cholinesterase inhibitory activity, demonstrating that sterically bulky substitution at the 3a position is poorly tolerated.

The alkaloid physostigmine (**1a**), isolated from the seeds of the *Calabar* bean (*Physostigma venenosum*),[1] is a potent reversible inhibitor of the cholinesterase enzymes, acetyl- and butyrylcholinesterase. It has been used clinically to treat glaucoma and appears beneficial in the treatment of dementia of the Alzheimer type,[2] which has an underlying cholinergic deficiency. Recently, extensive research has been undertaken to improve the selectivity of physostigmine for inhibition of acetyl- versus butyrylcholinesterase, to extend its duration of action, and to lower its toxicity to maximize its therapeutic potential. Chemical modifications have been focused on changing the carbamate side chain at C(5) and on substitutions at the *N*(1) and *N*(8) basic nitrogens,[3-8] with replacement of *N*(1) by other heteroatoms such as oxygen or sulfur,[9-11] and replacement of *N*(8) with sulfur[11] or a methylene group.[12] These studies have shown that a basic *N*(1)-atom need not be present in the tricyclic molecule for potent interaction with cholinesterase enzymes, and suggest that binding with either acetyl- or butyrylcholinesterase is through H-bonding interactions rather than through electrostatic attraction.[13] The potent inhibitory activities of 8-carbophysostigmine[12] and 8-thiaphysostigmine[11] analogs also have shown that a nitrogen in the *N*(8) position is not necessary.

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1) This paper is dedicated to Dr. Bernhard Witkop, Institute Scholar Emeritus, National Institutes of Health, Bethesda, Maryland, USA, at the occasion of his 80th birthday.

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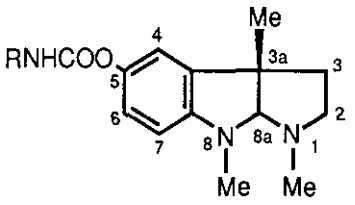
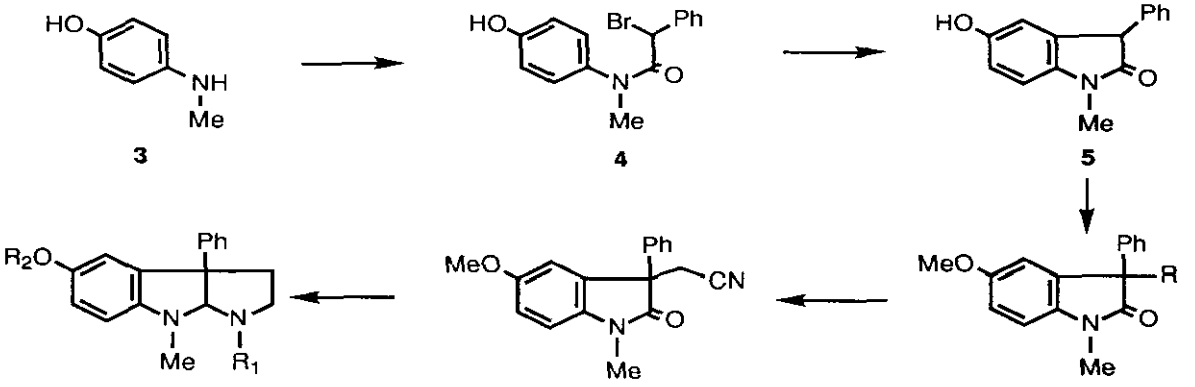


1a: R=Me, Physostigmine,
2a: R=Ph, Phenserine.

The development of phenserine (**2a**) [14] as an optimal drug candidate for Alzheimer's disease treatment is the result of these medicinal chemistry endeavours. However, the role of the 3a-methyl group remains unclear. As relacement of the methyl carbamate function of physostigmine with a phenyl carbamate resulted in the more potent and acetylcholinesterase-selective inhibitor, phenserine, it was therefore of interest to explore replacement of the 3a-methyl group with a sterically bulky and more lipophilic phenyl group to assess its effect on cholinesterase inhibition and further elucidate the structure/activity relation of this series of clinically interesting compounds. We report, herein, the syntheses and biological evaluation of the resulting novel agents.



Chemistry: The (±)-3a-phenyl analogs of physostigmine and phenserine were synthesized by modifying Julian's synthesis of physostigmine.[15,16] 4-Methylaminophenol (**3**) was *N*-acylated to give amide (**4**), which was converted to oxindole (**5**) by ring closure catalyzed by $AlCl_3$.[17] Methylation of **5** by Me_2SO_4 in aqueous KOH solution gave both 5-*O*-monomethylated product (**6**) and 5-*O*-, 3-*C*-dimethylated product (**7**), which could be separated by column chromatography followed by recrystallization giving 32% and 19% yields, respectively. Nitrile (**8**), which was obtained from **6** by phase transfer catalyzed *C*(3)-cyanomethylation, on reductive cyclization with Vitride [18,19] gave the amine (**9**). *N*(1)-Methylation of **9** gave the methyl ether (**10**), which was *O*-demethylated to the phenol (**11**) with BBr_3. Finally, (±)-3a-phenyl-3a-demethylphysostigmine (**12**) and (±)-3a-phenyl-3a-demethylphenserine (**13**) were obtained by treating **11** with methyl isocyanate and phenyl isocyanate, respectively.

Biological evaluation and discussion: Compounds (**12**) and (**13**) were tested for *in vitro* inhibitory activity of human erythrocyte acetylcholinesterase and plasma butyrylcholinesterase. The results, shown in Table 1, are compared with (-)-physostigmine (**1a**), (-)-phenserine (**2a**) and their racemic mixtures (**1**) and (**2**).[19] The activity of racemic mixtures in this series of compounds has invariably been shown to be about half the potency of the (3a*S*)-(-)-enantiomers, as was the case in this study. Our studies further demonstrate that both **12** and **13** lacked potent cholinesterase inhibitory activity. The loss of activity of compounds (**12**) and (**13**) is based on model predictions and is most likely due to the bulky perpendicularly situated phenyl group at C(3a), hindering the approach and close interaction/binding of the compounds with either acetyl- or butyrylcholinesterase to allow enzyme inhibition. This likely occurs despite the potential increased interaction between the lipophilic C(3a) phenyl and a known hydrophobic domain[20] close to the active site of the enzymes. Clearly, substitution of the 3a methyl group with a sterically bulky phenyl group in either physostigmine or phenserine is not well tolerated and is detrimental to inhibitory cholinesterase activity.

Table 1: *IC_{50} Values of Physostigmine Analogs Versus Human Erythrocyte Acetylcholinesterase and Plasma Butyrylcholinesterase* [a]

		IC_{50}(nM)	
No.	Compound	Acetylcholinesterase	Butyrylcholinesterase
1a	(-)-Physostigmine	27.9	16.0
2a	(-)-Phenserine	24.0	1300
1	(±)-Physostigmine	70.0	35.0
2	(±)-Phenserine	75.0	5610
12	(±)-3a-Phenyl-3a-demethylphysostigmine	9289	2734
13	(±)-3a-Phenyl-3a-demethylphenserine	>10,000	>25,000

[a] Minimum of 4 measurements per compound

EXPERIMENTAL

Melting points (uncorrected): Fisher-Johns apparatus; IR spectra (cm^{-1}): MIDIC FTIR instrument; ^1H-NMR (in $CDCl_3$ with Me_4Si as internal reference, δ ppm, J Hz): Varian XL-270 MHz spectrometer; MS (*m/z*) for chemical ionization (CI-MS): Finnigan-1015D mass spectrometer; MS (*m/z*) for electron impact (EI-MS): V.G. Micromass 7070 mass spectrometer; thin layer chromatography (silica gel GHLF, 250 mm): Analtech Inc.; column chromatography (silica gel GHLF, 250 mm): Merck 60 (230-400 mesh). Unless specified, all compounds were prepared and evaluated as racemic mixtures.

4-Hydroxy-N-methyl-N-(α-chloro-α-phenylacetyl)aniline (4): The sulfate of 4-methylaminophenol (**3**) (50.0 g, 0.145 mol) was dissolved in 500 mL of H_2O with heating. After adding 150 mL of saturated aqueous solution of $NaHCO_3$, the base was precipitated and extracted with ether (3x300 mL). The combined ether layers were washed with brine, dried over Na_2SO_4, to give, after evaporation of ether, the free base as crystals (35.0 g, 98 %). The free base was dissolved in 250 mL of THF, and 2-chloro-2-phenylacetyl chloride (20.0 g, 0.106 mol) in THF (20 mL) was added dropwise under cooling in an ice

bath. After the addition, the reaction mixture was refluxed for 1.5 h. Evaporation of solvent gave a residue which was dissolved in AcOEt (300 mL) and washed with 1N HCl (3x300 mL), brine (300 mL), and dried over Na_2SO_4. After removal of solvent *in vacuo*, 27 g (96% yield based on 2-chloro-2-phenylacetyl chloride) of a crystaline product was obtained which was used directly in the following reaction. The analytical sample was obtained by recrystallization from acetone as a colorless crystal. **4**: mp 138-140 °C; CI MS (*m/z*): 253 (M^++1); ^{1}H-NMR ($CDCl_3$): δ 7.38-7.10 (m, 5H, C3-Ph), 7.10-6.60 (m, 4H, Ph), 5.40 (s, 1H, OH), 3.27 (s, 3H, N1-CH_3), 1.75 (s, 1H, CH); IR (cm^{-1}): 3297 (OH), 1651 (CO).

1-Methyl-3-phenyl-5-hydroxyoxindole (5): A mixture of **4** (5.51 g, 20 mmol) and $AlCl_3$ (6.67 g, 49.7 mmol) was heated in an oil bath at approximately 90° C with manual stirring using a steel bar. After a short time, the reaction became vigorous and was accompanied with the formation of HCl. After solidification, another 6.67 g (49.7 mmol) of $AlCl_3$ was added and the temperature of the oil bath was raised to 180-185 °C. The solid mixture of reactants continued to be stirred until it became a dark liquid, and then was kept at this temperature for 1 h. The hot homogeneous melt was poured into a mortar, pulverized following cooling, and decomposed by addition of 50 g of crushed ice to give **5** as reddish crystals (4.7 g, 97 %). After recrystallization from ethanol, colorless crystals were obtained. **5**: mp 204-206 °C; CI MS (*m/z*): 240 (M^++1); ^{1}H-NMR ($CDCl_3$): δ 7.33-7.25 (m, 5H, C3-Ph), 7.18 (d, 1H, J=2.2 Hz, C4-H), 6.77 (dd, 1H, J=2.6, 8.4 Hz, C6-H), 6.72 (d, 1H, J=8.4 Hz, C7-H), 5.52 (b.s., 1H, OH), 4.54 (s, 1H, C3-H), 3.20 (s, 3H, N-CH_3); IR (cm^{-1}): 3227 (OH), 1678 (CO).

(±)-1-Methyl-3-phenyl-5-methoxyoxindole (6) and (±)-1,3-dimethyl-3-phenyl-5-methoxyoxindole (7): The oxindole (**5**) (3.59 g, 15 mmol) was dissolved in 5% KOH (25 mL) and Me_2SO_4 (2.88 g, 22.9 mmol) was added. The mixture was heated at 90° C for 1 h, cooled to rt, 5 % NaOH (25 mL) was added and the mixture was extracted with AcOEt (3x30 mL). The combined AcOEt layers were washed with 5 % NaOH (4x40 mL), dried over Na_2SO_4, and evaporated in vacuo. The residue was chromatographed on a silica gel column (ether:hexane=1:1) to give **7** (366 mg) as a light yellow syrup, **6** (470 mg) as corlorless crystals and a mixture of **6** and **7** (1.16 g) in a semicrystal form, from which 640 mg of pure **6** was obtained by recrystallization (AcOEt). **6**: mp 112-113 °C; EI MS (*m/z*): 253 (M^+), 238 (M-CH_3), 224, 210; CI MS (*m/z*): 254 (M^++1); ^{1}H-NMR ($CDCl_3$): δ 7.36-7.26 (m, 5H, C3-Ph), 7.22 (d, 1H, J=2.2 Hz, C4-H), 6.86 (dd, 1H, J=2.6, 8.4 Hz, C6-H), 6.80 (d, 1H, J=8.4 Hz, C7-H), 4.58 (s, 1H, C3-H), 3.75 (s, 3H, OCH_3), 3.20 (s, 3H, N-CH_3); IR (cm^{-1}): 1715 (CO).
7: EI MS (*m/z*): 267 (M^+), 252 (M-CH_3), 238, 224; CI MS (*m/z*): 268 (M^++1);^{1}H-NMR ($CDCl_3$): δ 7.30-7.20 (m, 5H, C3-Ph), 6.80 (d, 1H, J=2.2 Hz, C4-H), 6.72 (dd, 1H, J=2.6, 8.4 Hz, C6-H), 6.67 (d, 1H, J=8.4 Hz, C7-H), 3.78(s, 3H, OCH_3), 3.22 (s, 3H, N-CH_3), 1.78 (s, 3H, C3-CH_3); IR (cm^{-1}): 1711 (CO).

(±)-1-Methyl-3-phenyl-3-cyanomethyl-5-methoxyoxindole (8): Compound (**6**) (164 mg, 0.65 mmol) was dissolved in toluene (6 mL), and stirred with 50 % NaOH (2 mL) and benzyltrimethylammonium bromide (18 mg, 0.075 mmol) at 0° C under N_2 for 10 min, then chloroacetonitrile (54 mg, 0.72 mmol) was added.

The mixture was stirred at rt for 1 h, and ice cold water (15 mL) was added. The toluene layer was separated, and the aqueous layer was extracted with ether (2x10 mL). The combined organic layers were washed with brine (15 mL), dried over Na_2SO_4, and evaporated *in vacuo*. The residue was flash column chromatographed (CH_2Cl_2/MeOH=20/1) to give 175 mg (0.60 mg, 92 %) of colorless crystals. **8**: mp 138-139 °C; CI MS (*m/z*): 293 (M^++1); ^{1}H-NMRr ($CDCl_3$): δ 7.43-7.25 (m, 5H, C3-Ph), 7.12 (d, 1H, J=2.2 Hz, C4-H), 6.96 (dd, 1H, J=2.2, 8.8 Hz, C6-H), 6.88 (d, 1H, J=8.8 Hz, C7-H), 3.82 (s, 3H, OCH_3), 3.36 (d, 1H, J=16.7 Hz, CH_2CN, AB), 3.22 (s, 3H, N-CH_3), 3.05 (d, 1H, J=16.7 Hz, CH_2CN, AB).

*(±)-1,2,3,3a,8,8a-Hexahydro-3a-phenyl-8-methyl--5-methoxypyrrolo[2,3-b]indole ((±)-(3a)-Phenyl-3a-didemethyl-esermethole (**9**)):* Compound (**8**) (202 mg, 0.69 mmol) was dissolved in benzene (5.0 mL), and vitride (0.4 mL, 1.4 mmol) was added. The mixture was stirred under N_2 at rt for 1.5 h, and then extracted with 2N HCl (2x10 mL). The aqueous solution was washed with ether (2x10 mL), basified with 5 % Na_2CO_3, and extracted with CH_2Cl_2 (2x10 mL). After the removal of solvent *in vacuo*, the residue was column chromatographed (CH_2Cl_2/MeOH=20/1) to give 161 mg (0.57 mmol, 83 %) of a colorless oil. **9**: CI MS (*m/z*): 281 (M^++1); ^{1}H-NMR ($CDCl_3$): δ 7.36-7.29 (m, 5H, C(3a)-Ph), 6.67 (dd, 1H, J=2.6, 8.4 Hz, C6-H), 6.56 (d, 1H, J=2.6 Hz, C4-H), 6.34 (d, 1H, J=8.4 Hz, C7-H), 5.30 (s, 1H, C(8a)-H), 4.91 (s, 3H, OCH_3), 3.70 (s, 3H, N(8)-CH_3), 2.8 (s, 1H, N(1)-H), 2.80-2.35 (m, 2H, CH_2), 2.00-1.70 (m, 2H, CH_2).

*(±)-1,2,3,3a,8,8a-Hexahydro-1,8-dimethyl-3a-phenyl-5-methoxypyrrolo[2,3-b]indole ((±)-(3a)-Phenyl-3a-demethyl-esermethole (**10**)):* Compound (**9**) (170 mg, 0.60 mmol) was dissolved in CH_3OH (2.5 mL) and 37 % of CH_2O (0.34 mL) was added. The mixture was refluxed with stirring for 15 h. After cooling to 0°C, KBH_4 (100 mg, 2.0 mmol) was added in small portion, and then stirred at rt for 30 min. Evaporation of solvent gave a residue which was dissolved by 2N HCl (10 mL), stirred for 30 min, and then washed with petroleum ether to remove organic impurities. The aqueous solution was basified by slow addition of solid $NaHCO_3$ and extracted with ether (3x10 mL). Extracts then were dried over $MgSO_4$ and evaporated in vacuo. The residue was chromatographed (CH_2Cl_2/MeOH=20/1) to give 95 mg (0.32 mmol, 53 %) of colorless oil. **10**: CI MS (*m/z*): 295 (M^++1); ^{1}H-NMR ($CDCl_3$): δ 7.39-7.17 (m, 5H, C3a-Ph), 6.69 (dd, 1H, J=2.6, 8.4 Hz, C6-H), 6.53 (d, 1H, J=2.2 Hz, C4-H), 6.43 (d, 1H, J=8.8 Hz, C7-H), 4.44 (s, 1H, C8a-H), 3.70 (s, 3H, OCH_3), 2.94 (s, 3H, N8-CH_3), 2.52 (s, 3H, 1H, N1-CH_3), 2.90-2.2 (m, 4H, 2xCH_2).

*(±)-1,2,3,3a,8,8a-Hexahydro-1,8-dimethyl-3a-phenyl-5-methoxypyrrolo[2,3-b]indol-5-ol ((±)-(3a)-Phenyl-3a-demethyleseroline (**11**)):* Compound (**10**) (90 mg, 0.31 mmol) was dissolved in CH_2Cl_2 (5 mL) and BBr_3 (0.2 mL) was added. The mixture was stirred at rt for 15 h. Thereafter, MeOH (5 mL) was added dropwise. The solvent was removed *in vacuo*, H_2O (10 mL) was added, the aqueous solution was basified by slow addition of solid $NaHCO_3$, and then extracted with ether (3x10 mL). The extracts were dried over $MgSO_4$ and evaporated *in vacuo*. The residue was flash column chromatographed

(CH_2Cl_2/MeOH=20/1) to give 59 mg (0.21 mmol, 68 %) of light pink foam. **11**: CI MS (*m/z*): 281 (M^++1); ^{1}H-NMR ($CDCl_3$): δ 7.40-7.19 (m, 5H, C(3a)-Ph), 6.70 (dd, 1H, J=2.6, 8.4 Hz, C6-H), 6.53 (d, 1H, J=2.6 Hz, C4-H), 6.41 (d, 1H, J=8.4 Hz, C7-H), 5.12 (s, 1H, C8a-H), 4.48 (s, 1H, OH), 2.94 (s, 3H, N8-CH_3), 2.56 (s, 3H, 1H, N1-CH_3), 2.90-2.2 (m, 4H, 2xCH_2).

(±)-1,2,3,3a,8,8a-Hexahydro-1,8-dimethyl-3a-phenyl-5-methoxypyrrolo[2,3-b]indol-5-yl N-methylcarbamate ((±)-(3a)-Phenyl-3a-demethylphysostigmine (12)): Compound (**11**) (29 mg, 0.10 mmol) was dissolved in ether (5 mL) and Na (approximately 1 mg) was added. The mixture was stirred at rt for 10 h, and methyl isocyanate (6.1 mg, 0.11 mmol) was added. The mixture was stirred at rt overnight, and the solvent removed *in vacuo*. The residue was flash column chromatographed (CH_2Cl_2/MeOH=20/1) to give 16 mg (0.047 mmol, 47 %) of colorless foam. **12**: CI MS (*m/z*): 338 (M^++1); ^{1}H-NMR ($CDCl_3$): δ 7.40-7.20 (m, 5H, C3a-Ph), 6.90 (dd, 1H, J=2.2, 8.4 Hz, C6-H), 6.73 (d, 1H, J=2.2 Hz, C4-H), 6.48 (d, 1H, J=8.4 Hz, C7-H), 4.90 (s, 1H, NH), 4.60 (br s, 1H, C8a-H), 2.94 (s, 3H, N8-CH_3), 2.89 (d, 3H, J=4.1 Hz, CH_3NH), 2.80-2.70 (m, 2H, CH_2), 2.56 (s, 3H, 1H, N1-CH_3), 2.40-2.30 (m, 2H, CH_2).

(±)-1,2,3,3a,8,8a-Hexahydro-1,8-dimethyl-3a-phenyl-5-methoxypyrrolo[2,3-b]indol-5-yl N-phenylcarbamate ((±)-(3a)-Phenyl-3a-demethylphenserine (13)): Prepared from compound (**11**) as described for the preparation of **12** but with the use of phenyl isocyanate, yield 48 %. **13**: CI MS (*m/z*): 400 (M^++1); ^{1}H-NMR ($CDCl_3$): δ 7.42-7.07 (m, 10H, 2xPh), 6.94 (dd, 1H, J=2.2, 8.4 Hz, C6-H), 6.75 (d, 1H, J=2.2 Hz, C4-H), 6.46 (d, 1H, J=8.4 Hz, C7-H), 5.10 (s, 1H, NH),4 .62 (br s, 1H, C8a-H), 3.00 (s, 3H, N8-CH_3), 2.80-2.70 (m, 2H, CH_2), 2.56 (s, 3H, 1H, N1-CH_3), 2.40-2.30 (m, 2H, CH_2).

Biological Assay. Freshly collected human blood was centrifuged (6000xg, 10 min, at 4°C), the plasma was separated and diluted 1/125 with 0.1 M Na_3PO_4 (pH 7.4). Erythrocytes were washed three times in isotonic saline, and lysed by the addition of 9 volumes of 0.1 M Na_3PO_4 containing 0.5 % Triton-X (Sigma Chemical Co., St. Louis, MO) (pH 7.4 on ice for 30 min). This then was diluted with 19 further volumes of 0.1 M Na_3PO_4, (pH 7.4) to final dilution of 1/200. Acetyl-β-methylthiocholine (0.5 mM) (Sigma) and *S*-butyrylthiocholine (0.5 mM) (Sigma) were used as specific substrates for the assay of acetyl- and butyrylcholinesterase, respectively. For each cholinesterase preparation, 25 μL of substrate and 25 μL of enzyme were added separately to a final incubation volume of 0.75 mL.

All compounds initially were dissolved in Tween 80/EtOH (3/1, v/v, 75 μL total volume) and were then diluted with 0.1 M Na_3PO_4, (pH 8.0) in half log-increments to a final concentration range of between $1x10^{-5}$ M and $0.3x10^{-9}$ M. Finally, they were preincubated with enzyme (30 min at 21°C) prior to addition of substrates. The Tween 80/EtOH was diluted to in excess of 1:1000 and did not affect either acetyl- or butyrylcholinesterase activity, as determined in prior studies with physostigmine.

Following a 25 min incubation at 37° C with respective substrates for acetyl- and butyrylcholinesterase and 5,5'-dithiobis-2-nitrobenzoic acid (0.5 mmol/L), the absorbance of a yellow thionitrobenzoate anion product was measured with a spectrophotometer set to 412 nm wavelength. Nonspecific substrate hydrolysis was determined under conditions of complete enzyme inhibition (by the addition of excess physostigmine, $1x10^{-5}$ M), and the associated change in absorbance was subtracted from that observed with the experimental compounds (**1**,**1a**,**2**,**2a**,**12**,**13**). Furthermore, the activity of each compound was assessed alongside that of physostigmine, as an external standard, whose activity we have previously reported.[5,6,9,10,21]

For determination of an IC_{50} value for each compound, the enzyme activity at each concentration was expressed as a percent of that determined in the absence of compound. This then was transformed into a logit format, where logit = In(% activity / [100 - % activity]), and was plotted as a function of the log concentration of the compound. IC_{50} values (i.e., logit = In(50/[100-50]=0) were determined only when correlation coefficients of less than -0.99 were achieved, and when more than 50 % inhibition was achieved from duplicate samples. Each compound was analyzed on at least 4 occasions, in duplicate. The IC_{50} values are listed in Table 1.

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