DOI 10.1007/s11094-019-02035-7
Pharmaceutical Chemistry Journal, Vol. 53, No. 6, September, 2019 (Russian Original Vol. 53, No. 6, June, 2019)

SYNTHESIS AND ANTICHOLINESTERASE ACTIVITY EVALUATION OF ASYMMETRIC AZINES

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Original article submitted November 19, 2018.

Azines or di-imines are compounds containing R_1R_2C=N-N=CR_3R_4 fragment in its structure. These compounds have received special attention due to their importance as intermediates in the synthesis of drugs and substances with diverse pharmacological activities. In this research, twelve compounds derived of asymmetric azines of aryl aldehydes and aryl ketones were synthesized by microwave assisted irradiation, and their ability to inhibit acetylcholinesterase (AChE) enzyme was tested using the Ellman method. Among the evaluated azines, five compounds were able to inhibit AChE, especially compounds (7E, 8E)-1-benzylidene-2-(1-(4-nitrophenyl)ethylidene)hydrazine (**7**) and (7E,8E)-2-(1-(4-nitrophenyl)ethylidene)-1-((pyridin-2-yl)methylene)-hydrazine (**14**), which showed percentage inhibition of 18.34% and 17.43% at concentrations of 22.46 and 10 μM, respectively.

Keywords: azine, synthesis, microwave assisted irradiation, inhibition, acetylcholinesterase.

1. INTRODUCTION

Azines or di-imines are compounds containing R_1R_2C=N-N=CR_3R_4 fragment in the structure, which are usually synthesized by reactions between a primary amine and carbonyl groups of aldehydes and/or ketones [1, 2]. Depending on the nature of R substituents, the azines can be classified into symmetric ($R_1R_2 = R_3R_4$), asymmetric ($R_1R_2 \neq R_3R_4$), aldazines ($R_1 = R_3 = H$), ketazines ($R_1R_2R_3R_4 \neq H$), mixed azines ($R_1 = H$; R_2 R_3 $R_4 \neq H$), and aromatic, aliphatic, cyclic and acyclic azines [3]. For example, Fig. 1 shows the structure of aromatic hydrazines.

The classical method to obtain azines is based on the reaction of aldehydes with hydrazine. From the synthetic point of view, there are two strategies in obtaining these compounds: (1) heating the hydrazine with aldehydes and/or ketones in solvents such as ethanol, methanol, tetrahydrofuran, butanol. and glacial acetic acid and (2) the condensation reaction between hydrazine derivatives and aldehydes and/or ketones catalyzed by acids or bases [4].

In recent years, some series of azines have been prepared by applying microwave assisted irradiation which can dramatically reduce the preparation time and provide good yields [5, 6]; on the other hand, it is in consonance with green chemistry. These compounds have received special attention due to their importance as intermediates in the synthesis of drugs and substances with diverse pharmacological properties. The known biological activities include, among others, anticonvulsant, antibacterial, antiparasitic, insecticidal, anti-inflammatory, antioxidant and anti-tumor [7 – 15].

In view of this broad spectrum of activities described in literature and in continued search for compounds capable of inhibiting acetylcholinesterase (AChE) enzyme that plays a key role in the evolution of Alzheimer's disease, in this paper we report the synthesis, characterization and evaluation of

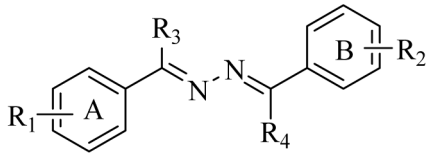

Fig. 1. Aromatic azines.

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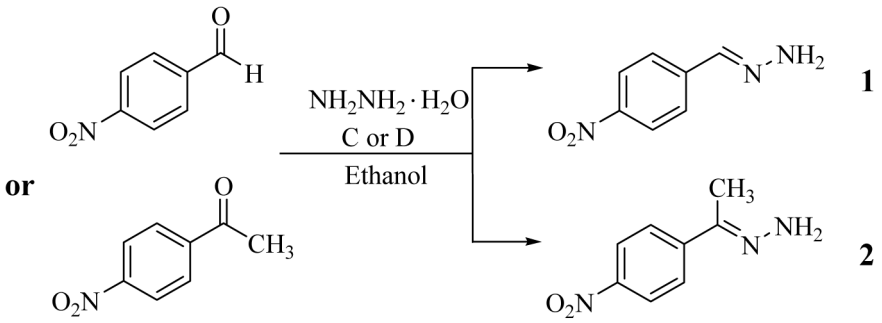


Scheme 1. Synthesis of hydrazones **1** and **2.**

the anticholinesterase activity of a series of asymmetric azine derivatives of aryl aldehydes and aryl ketones.

2. MATERIALS AND METHODS

All reagents were purchased from Sigma-Aldrich and used without additional purification. Thin layer chromatography (TLC) analysis was performed on Merck silica gel 60F254 plates using hexane/ethyl acetate as the solvent and iodine for developing TLC plates. Microwave irradiation was carried out by using Discover DC-7196 CEM Corporation reactor. The melting points were determined in open capillaries using Microquímica MQAPF-301 electrical apparatus and were uncorrected. The characterization was performed by gas chromatograph/mass spectrometry (GC-MS), infrared (IR) spectrophotometry, and nuclear magnetic resonance (NMR) spectroscopy.

The IR spectra were recorded in KBr pellets on the FT-IR PerkinElmer Spectrum Frontier instrument. The NMR spectra were recorded on Bruker Avance 500 instrument working at 500 MHz (^{1}H) and 125 MHz (^{13}C) frequencies, using $CDCl_3$-*d* as the solvent and tetramethylsilane (TMS) as an internal standard with the chemical shifts (δ values) expressed in ppm units. The mass spectra were recorded in Shimadzu GC-MS QP-2010 Ultra spectrometer.

3. EXPERIMENTAL SECTION

3.1. Synthesis

Twelve derived asymmetric azines of aryl aldehydes and aryl ketones were synthesized, with *p*-nitro substituent on A ring and other groups on B ring (Fig. 1). The synthesis of these compounds passed via the initial formation of hydrazones **1** and **2** (Scheme 1) obtained by the reaction between 4-nitrobenzaldehyde or 4-nitroacetophenone with hydrazine in ethanol under conditions C or D.

The following series of four azines were synthesized by the reaction of hydrazones **1** or **2** with various benzaldehydes or acetophenones substituted under different heating conditions (E or F) in ethanol according to Scheme 2.

Synthesis of (E)-1-(4-nitrobenzylidene)hydrazine 1 (C). Hydrazine monohydrate (1 mmol, 40 μL) and 4-nitrobenzaldehyde (1 mmol, 151 mg) were dissolved in anhydrous ethanol and the solution was stirred at room temperature for 10 min. The formation of hydrazone was confirmed by GC-MS data.

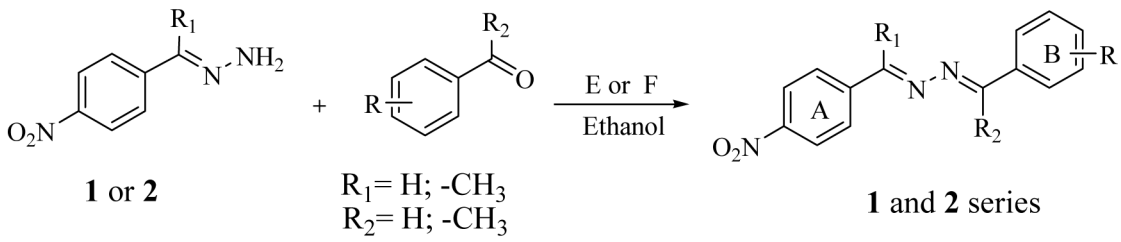
Synthesis of (E)-1-(1-(4-nitrophenyl)ethylidene)hydrazine 2 (D). Hydrazine hydrate 70% solution (1 mmol) was added to 5 ml of anhydrous ethanol, followed by adding of 4-nitroacetophenone (1 mmol, 165 mg). The mixture was heated in a microwave reactor at 300 W in two cycles: 2.5 min at 120°C and 1.5 min at 100°C. Then, the reaction mixture was cooled, and the precipitate was filtered and washed with ethanol.

Synthesis of Series 1 (compounds 3 – 6) (E). Equivalent amounts (1 mmol) of hydrazone **1** and various substituted benzaldehyde were added to a reaction tube with ethanol (2 mL) and kept under constant stirring for 2 h at room temperature. The precipitate was filtered and washed with cold ethanol.

Synthesis of Series 2 (compounds 7 – 14) (F). Equivalent amounts (1 mmol) of hydrazone **2** and various substituted benzaldehydes were added to a reaction tube containing ethanol (2 mL). The tube was sealed and the mixture was heated in a microwave reactor at 300W for 3 cycles: first being 1.5 min at 80°C, followed by two cycles of 2.5 min at 100°C. The precipitate was filtered and washed with cold ethanol.

(7E,8E)-2-(4-nitrobenzylidene)-1-benzylidenehydrazine (3): $C_{14}H_{11}N_3O_2$ (253.09 g/mol); yield 58.8%; m.p. 158.6°C; IR (KBr; ν, cm^{-1}): 1600 (C=N), 1520 (NO_2); ^{1}H NMR (500 MHz, $CDCl_3$; δ, ppm): 7.47 (m, 3H, Ar-H), 7.87 (d, 2H, Ar-H), 8.02 (d, 2H, Ar-H), 8.31 (d, 2H, Ar-H), 8.68 (s, 1H, N=CH), 8.7 (*s*, 1H, N=CH); ^{13}C NMR (125 MHz, $CDCl_3$; δ, ppm): 124.04, 128.87, 129.13, 131.79, 133.59, 136.59, 140.03 and 163.57 (Ar), 159.21 (N=C); MS (*m/z*): 253 $[M + H]^+$, 176, 131, 104, 77.

(7E,8E)-1-(4-methylbenzylidene)-2-(4-nitrobenzylidene)hydrazine (4): $C_{15}H_{13}N_3O_2$ (267.1 g/mol); yield 60.37%; m.p. 163.8°C; **IR** (KBr; ν, cm^{-1}): 1600 (C=N), 1520 (NO_2);



Scheme 2. Synthesis of asymmetric azines.

^{1}H NMR (500 MHz, $CDCl_3$; δ, ppm): 2.43 (s, 3H, CH_3), 7.28 (d, 2H, Ar-H), 7.76 (d, 2H, Ar-H), 8.0 (d, 2H, Ar-H), 8.31 (d, 1H, Ar-H), 8.66 (s, 1H, N=CH), 8.69 (s, 1H, N=CH); ^{13}C NMR (125 MHz, $CDCl_3$; δ, ppm): 21.9 (CH_3), 124.21, 128.79, 129.1, 129.7, 129.89, 140.33, 142.67 and 164 (Ar), 159.21 (N=C); MS (*m/z*): 267 $[M + H]^+$, 176, 145, 118, 91.

(7E,8E)-1-(4-ethylbenzylidene)-2-(4-nitrobenzylidene)-hydrazine (5): $C_{16}H_{15}N_3O_2$ (281.3 g/mol); yield 78%; m.p. 162.5°C; IR (KBr; ν, cm^{-1}): 1600 (C=N), 1510 (NO_2); ^{1}H NMR (500 MHz, $CDCl_3$; δ, ppm): 1.28 (t, 3H, CH_3), 2.73 (q, 2H, –CH_2), 7.3 (d, 2H, Ar-H), 7.79 (d, 2H, Ar-H), 8.0 (d, 2H, Ar-H), 8.31 (d, 2H, Ar-H), 8.66 (s, 1H, N=CH), 8.69 (s, 1H, N=CH); ^{13}C NMR (125 MHz, $CDCl_3$; δ, ppm): 15.25 (CH_3), 29 (CH_2CH_3), 123.99, 128.48, 128.78, 129.03, 131.18, 140.11, 148.72 and 163.7 (Ar), 158.29 (N=C); MS (*m/z*): 281 $[M + H]^+$, 252, 176, 159, 132.

(7E,8E)-2-(4-nitrobenzylidene)-1-((pyridin-2-yl)methylene)hydrazine (6): $C_{13}H_{10}N_4O_2$ (254.24 g/mol); yield 34%; m.p. 159 °C; IR (KBr; ν, cm^{-1}): 1600 (C=N), 1520 (NO_2), 1460 (Py); ^{1}H NMR (500 MHz, $CDCl_3$; δ, ppm): 7.38 (m, 1H, Ar), 7.81 (m, 1H, Ar), 8.04 (d, 2H, Ar), 8.1 (d, 1H, Ar), 8.32 (d, 2H, Ar), 8.69 (s, 1H, N=CH), 8.7 (s, 1H, N=CH), 8,75 (d, 1H, Ar); ^{13}C NMR (125 MHz, $CDCl_3$; δ, ppm): 122.66, 124.05, 125.35, 129.38, 136.67, 149.39, 150.1, 150.17 and 162.85 (Ar), 159.8 (N=C); MS (*m/z*): 254 $[M + H]^+$, 176, 105, 79.

(7E,8E)-1-benzylidene-2-(1-(4-nitrophenyl)ethylidene)hydrazine (7): $C_{15}H_{13}N_3O_2$ (267.28 g/mol); yield 68%; m.p. 147°C; IR (KBr; ν, cm^{-1}): 1598 (C=N), 1520 (NO_2); ^{1}H NMR (500 MHz, $CDCl_3$; δ, ppm): 2.55 (s, 3H, CH_3), 7.47 (m, 3H, Ar), 7.86 (d, 2H, Ar), 8.08 (d, 2H, Ar), 8.28 (d, 2H, Ar), 8.43 (s, 1H, N=CH); ^{13}C NMR (125 MHz, $CDCl_3$; δ, ppm): 15.26 (CCH_3), 123.57, 127.7, 128.5, 128.81, 131.25, 134.3, 144.11 and 162.3 (Ar), 148.66 and 159.02 (N=C); MS (*m/z*): 267 $[M + H]^+$, 190, 104, 77.

(7E,8E)-1-(4-methylbenzylidene)-2-(1-(4-nitrophenyl)-ethylidene)hydrazine (8): $C_{16}H_{15}N_3O_2$ (281,12 g/mol); yield 74%; m.p. 122.9°C; IR (KBr; ν, cm^{-1}): 1600 (C=N), 1520 (NO_2); ^{1}H NMR (500 MHz, $CDCl_3$; δ, ppm): 2.42 (s, 3H, CH_3), 2.55 (s, 3H, CH_3), 7.27 (d, 2H, Ar), 7.77 (d, 2H, Ar), 8.08 (d, 2H, Ar), 8.27 (d, 2H, Ar), 8.42 (s, 1H, N=CH); ^{13}C NMR (125 MHz, $CDCl_3$; δ, ppm): 15.22 (CCH_3), 21.62 (Ar-CH_3), 123.57, 127.66, 128.55, 129.56, 131.64, 141.83, 144.24 and 162.18 (Ar), 148.62 and 159.29 (N=C); MS (*m/z*): 281 $[M + H]^+$, 266, 190, 159, 118.

(7E,8E)-1-(4-isopropylbenzylidene)-2-(1-(4-nitrophenyl)ethylidene)hydrazine (9): $C_{18}H_{19}N_3O_2$ (309.15 g/mol); yield 65.5%; m.p. 96.9 °C; IR (KBr; ν, cm^{-1}): 1600 (C=N), 1510 (NO_2); ^{1}H NMR (500 MHz, $CDCl_3$; δ, ppm): 1.3 (d, 6H, CH_3), 2.56 (s, 3H, CH_3), 2.99 (m, 1H, CH), 7.99 (d, 2H, Ar), 8.07 (d, 2H, Ar), 8.28 (d, 2H, Ar), 8.3 (d, 2H, Ar), 8.43 (s, 1H, N=CH); ^{13}C NMR (125 MHz, $CDCl_3$; δ, ppm): 15.24 (CH_3), 23.79 (CH_3), 34.26 ($CH(CH_3)_2$), 123.59, 126.98, 127.5, 128.7, 132, 144.24 and 162,14 (Ar), 148.61, 152.7 and 159.15 (N=C); MS (*m/z*): 309 $[M + H]^+$, 294, 190, 91.

(7E,8E)-1-(4-methoxybenzylidene)-2-(1-(4-nitrophenyl)ethylidene)hydrazine (10): $C_{16}H_{15}N_3O_3$ (297.31 g/mol); yield 44,3%; m.p. 134.3°C; IR (KBr; ν, cm^{-1}): 1600 (C=N), 1510 (NO_2), 1250 (O–C); ^{1}H NMR (500 MHz, $CDCl_3$; δ, ppm): 2.56 (s, 3H, CH_3), 3.88 (s, 3H, CH_3), 6.98 (d, 2H, Ar), 7.82 (d, 2H, Ar), 8.08 (d, 2H, Ar), 8.28 (d, 2H, Ar), 8.42 (s, 1H, N=CH); ^{13}C NMR (125 MHz, $CDCl_3$; δ, ppm): 15.21 (CH_3), 58.44 (OCH_3), 114.34, 123.59, 127.11, 127.66, 130.33, 144.36, 160.65 and 162.24 (Ar), 148.56 and 159.21 (N=C); MS (*m/z*): 297 $[M + H]^+$, 208, 190, 134, 104, 77.

(7E,8E)-1-(3,4,5-trimethoxybenzylidene)-2-(1-(4-nitrophenyl)ethylidene)hydrazine (11): $C_{18}H_{19}N_3O_5$ (357.36 g/mol); yield 67.4; m.p. 142.5°C; IR (KBr; ν, cm^{-1}): 1590 (C=N), 1510 (NO_2), 1230 (O–C); ^{1}H NMR (500 MHz, $CDCl_3$; δ, ppm): 2.56 (s, 3H, CH_3), 3.92 (s, 3H, CH_3), 3.95 (s, 6H, CH_3), 7.1 (s, 2H, Ar), 8.07 (d, 2H, Ar), 8.29 (d, 2H, Ar), 8.33 (s, 1H, N=CH); ^{13}C NMR (125 MHz, $CDCl_3$; δ, ppm): 15.28 (CCH_3), 56.26 (OCH_3), 60.98 (OCH_3), 105.66, 123.57, 127.66, 129.64, 141.08, 144.08, 153.57 and 158.95 (Ar), 148.6 and 158.87 (N=C); MS (*m/z*): 357 $[M + H]^+$, 326, 208, 190, 131.

(7E,8E)-1-(3-methoxy,4-hidroxybenzylidene)-2-(1-(4-nitrophenyl)ethylidene)hydrazine (12): $C_{16}H_{15}N_3O_4$ (313.31 g/mol); yield 67%; m.p. 144.9°C; IR (KBr; ν, cm^{-1}): 3500 (OH), 1590 (C=N), 1520 (NO_2), 1200 (O–C); ^{1}H NMR (500 MHz, $CDCl_3$; δ, ppm): 1.58 (s, 1H, OH), 2.56 (s, 3H, CH_3), 3.99 (s, 3H, CH_3), 5.98 (s, 1H, Ar), 6.98 (d, 1H, Ar), 7.52 (d, 1H, Ar), 8.06 (d, 2H, Ar), 8.26 (d, 2H, Ar), 8.37 (s, 1H, N=CH); **^{13}C NMR** (125 MHz, $CDCl_3$; δ, ppm): 15.22

(CCH_3), 56,06 (OCH_3), 108.74, 114.49, 123.59, 124.55, 127.63, 144.29, 147, 147.7 and 161.95 (Ar), 148.97 and 159.41 (N=C); MS (*m/z*): 313 $[M + H]^+$, 296, 190, 150.

(7E,8E)-1-(4-N,N-dimethylbenzylidene)-2-(1-(4-nitrophenyl)ethylidene)hydrazine (13): $C_{17}H_{18}N_4O_2$ (310.35 g/mol); yield 68,4%; m.p. 194.9°C; IR (KBr; ν, cm^{-1}): 1600 (C=N), 1525 (NO_2), 1500 (C–N); ^1H NMR (500 MHz, $CDCl_3$; δ, ppm): 2.58 (s, 3H, CH_3), 3.06 (s, 6H, CH_3), 6.72 (d, 2H, Ar), 7.76 (d, 2H, Ar), 8.06 (d, 2H, Ar), 8.27 (d, 2H, Ar), 8.43 (s, 1H, N=CH); ^{13}C NMR (125 MHz, $CDCl_3$; δ, ppm): 15 (CCH_3), 40.12 (NCH_3), 111.68, 122.05, 123.51, 127.48, 130.32, 144.73, 160 and 161.33 (Ar), 152.53 and 160.68 (C=N); MS (*m/z*): 310 $[M + H]^+$, 190, 147, 77.

(7E,8E)-2-(1-(4-nitrophenyl)ethylidene)-1-((pyridin-2-yl)methylene)hydrazine (14): $C_{14}H_{112}N_4O_2$ (268.1 g/mol); yield 77.9%; m.p. 157 – 158°C; IR (KBr; ν, cm^{-1}): 1600 (C=N), 1510 (NO_2); ^1H NMR (500 MHz, $CDCl_3$; δ, ppm): 2.5 (s, 3H, CH_3), 7.37 (m, 1H, Py), 7.8 (m, 1H, Py), 8.09 (d, 2H, Ar), 8.15 (d, 1H, Py), 8.28 (d, 2H, Ar), 8.38 (s, 1H, Py), 8.7 (s, 1H, N=CH); ^{13}C NMR (125 MHz, $CDCl_3$; δ, ppm): 15.43 (CCH_3), 121.87, 123.63, 124.97, 127.86, 136.63, 143.71, 153.23, 157.8 (Ar), 148.77 and 161.64 (C=N); MS (*m/z*): 268 $[M + H]^+$, 239, 190, 144, 78.

3.2. In Vitro Anti-Acetylcholinesterase Activity

The ability of the synthesized compounds to inhibit AChE enzyme was tested using the Ellman method as modified by Rhee, et al. [16]. Tests were performed in 96-well microplates with automatic reader. To each well in microplate were added 125 μL of 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) 3 mM in Tris buffer at pH 8 (50 μL) containing 0.1% of bovine serum albumin and 25 μL of 1 mM sample solution in methanol. The absorbance was monitored at 412 nm every 10 sec during 230 sec. After this period, 25 μL of AChE enzyme aqueous solution 0.226 U/mL were added and the absorbance was monitored at 412 nm every 10 sec during 230 sec. Physostigmine (eserine) was used as positive control. All reactions were carried out in triplicate.

The enzyme activity was expressed as percentage inhibition (%I) of AChE enzyme, by comparing the rates of test reactions in microplate wells with samples and test reaction using buffer without any inhibitor. The calculation was made according to the following formula:

$$\%I = [V\,(\text{enzyme without sample}) - V\,(\text{enzyme with sample})] / [V\,(\text{enzyme without sample})] \times 100.$$

Any increase in the absorbance before addition of the enzyme due to the spontaneous hydrolysis of the substrate is properly corrected by subtracting the rate of the reaction before addition of the enzyme, obtained after addition of the enzyme. The samples were analyzed in triplicate [16, 17]. Results presented in this study are the average of three replicates ($n = 3$) and expressed as mean with standard error (±SEM). Statistical differences between the treatment and control were analyzed by ANOVA test and $P < 0.05$ was considered to be significant ($^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

4. RESULTS AND DISCUSSION

4.1. Synthesis of Azines

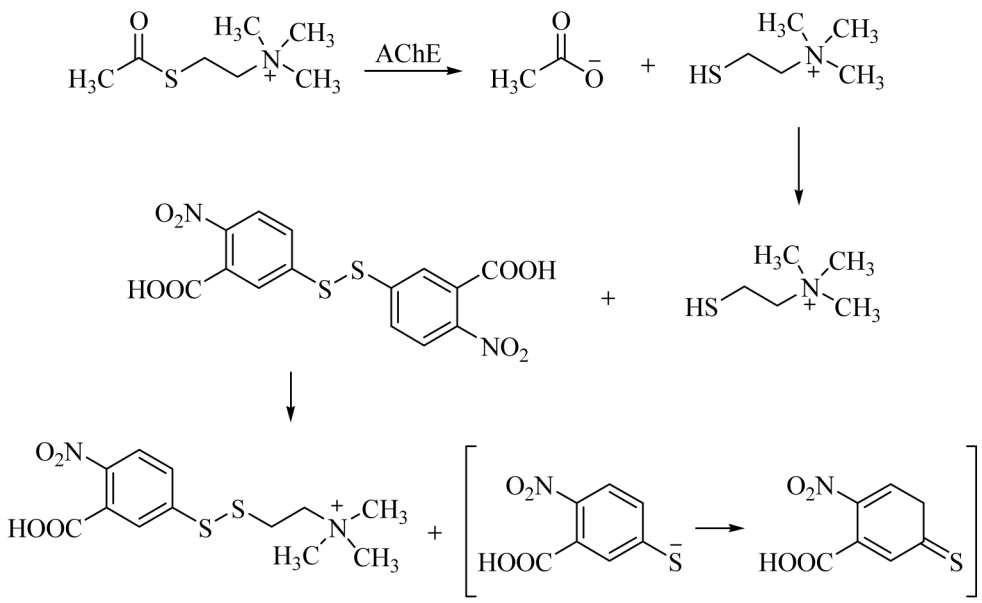
A total of 14 asymmetric azines were synthesized. These compounds were obtained by reaction between aromatic aldehydes/ketones and hydrazine according to procedures C, D, E and F. The nitro group is fixed at *para* position on ring A and the substituents are varied on the other ring B (Scheme 2). The structures of the synthesized compounds as well as the percentage conversion of the reactants to expected products are presented in Table 1.

In comparison of the percentage formation of asymmetric azines, **Series 2** showed higher conversion to expected products than **Series 1**. This fact can be explained by the greater tendency of symmetric hydrazone to form dimers (**Series 1**), due to their high reactivity. Hydrazones can react with each other forming dimers and releasing hydrazine in the reaction medium. The released hydrazine can then react with a second aldehyde present in the medium to form a new hydrazone and therefore a new dimer [18]. These competing reactions decrease the percentage of formation of the expected product, which may therefore explain the smaller percentage of formation of asymmetric azines in **Series 1**. In such cases, addition of the compound was provided for the formation of corresponding dimers.

Few reports like the synthesis of asymmetric compounds obtained in this study were found in the literature and none described the use of microwave assisted irradiation as the heating source. However, the synthesis of symmetric azines

TABLE 1. Synthesis of Asymmetric Azines under Microwave Irradiation

Compound	B ring	R_1	R_2	Procedure	% Conversion
3	Ph	H	H	E	62.8
4	4-CH_3-C_6H_4	H	H	E	70.3
5	4-CH_2CH_3-C_6H_4	H	H	E	55.0
6	2-pyridine	H	H	E	67.2
7	Ph	CH_3	H	F	98.1
8	4-CH_3-C_6H_4	CH_3	H	F	94.0
9	4-$CH(CH_3)_2$-C_6H_4	CH_3	H	F	97.0
10	4-OCH_3-C_6H_4	CH_3	H	F	89.0
11	3,4,5-OCH_3	CH_3	H	F	79.0
12	3-OCH_3, 4-OH	CH_3	H	F	90.3
13	4-NMe_2-C_6H_4	CH_3	H	F	98.7
14	2-pyridine	CH_3	H	F	93.15



Scheme 3. Sequence of reactions according to the Ellman method

has frequently been described both under conventional heating conditions and by microwave assisted irradiation using different reagents [3 – 5, 19]. A good example is this series was reported by Kurteva, et al. [3], where 21 asymmetric azines were synthesized by reaction of aromatic aldehydes and hydrazine sulfate in ethanol/DMF, with yields between 84 and 98%. The highest yield in obtaining dimers is expected since it involves only one step instead of two as in the preparation of asymmetric azines.

TABLE 2. Percentage Inhibition of AChE by Synthetized Azines

Compound	% Inhibition[a]	Concentration (μM)
3	0.50 ± 0.03**	100
4	NI[b]	10
5	NI[b]	10
6	NI[b]	10
7	18.34 ± 1.02***	22.5
8	NI[b]	100
9	NI[b]	10
10	NI[b]	100
11	NI[b]	100
12	2.99 ± 0.11**	100
13	15.56 ± 0.87**	100
14	17.43 ± 0.93*	10
Physostigmine	27.23 ± 0.82	100

[a] Data are expressed as mean ± SEM (triplicate); [b] NI = no inhibition; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

4.2. In Vitro Activity Assays

The ability to inhibit AChE enzyme by synthesized compounds was evaluated *in vitro*. Compounds with several substituents at different positions (*ortho*, *meta* and *para*) of ring B were evaluated in respect of the influence of structural factors on the activity. Initially, the objective was to verify dependence of the activity on alkyl radicals in ring B (compounds **3**, **4**, **5**), as well as the effect of changes caused by the insertion of a methyl group at carbon adjacent to ring A (compounds **7**, **8** and **9**). Since compounds **6** and **14** were selected for bioisosteric substitution (CH → N) at the *ortho* position of ring B over compounds **3** and **7**. The analysis of the influence of oxygen radicals (-OH and -OCH_3) determined the choice of compounds **10**, **11** and **12**. Finally, compound **13** was chosen to introduce amino substituent. The tests were carried out based on the Ellman method [17]. In this case, the enzyme hydrolyzes a substrate, resulting in the production of thiocholine that reacts with 5,5'-dithio-bis(2-nitrobenzoic acid, known as the Ellman reagent, to produce 2-nitrobenzoato-5-mercaptothiocholine/5-thio-2-nitrobenzoate which can be detected at 412 nm (Scheme 3). The absorbance indicating inhibition of the enzyme decreases when substances capable of inhibiting AChE are added.

This photometric method requires high solubility of all reactants and especially aqueous samples, since the solutions are prepared in aqueous buffers. This problem was partially solved by Rhee, et al. [16] by adapting a method that allowed using non-aqueous solutions and monitoring the production of colored marker compounds in 96-well plates with

microplate reader. This adaptation offered a very sensible and efficient method due to analysis of many substances in a short time at very low concentrations, making it the most widely used assessment of natural and synthetic compounds [20]. The percentage of AChE inhibition by azines and the maximum concentrations of drugs tested are shown in Table 2.

The anti-AChE activity tests revealed low levels of the enzyme inhibition or lack of activity. Compounds **3**, **7**, **12**, **13** and **14** showed the ability to inhibit the enzyme *in vitro*. Among these, the maximum percentages of inhibition were shown by com pounds **7** (18.24%) and **13** (17.53%), which did not have substituents on ring B. Compound **13** (with dimethylamino group in *p*-position) exhibited 15.56% enzyme inhibition. Compounds **3** and **12** showed percentage inhibition of 0.5 and 2.99%, respectively, which were at the lowest inhibition level and could can be classified as false positive [21, 22].

Further studies, like IC_{50} and bioavailability estimations are needed to establish the best structure that influences the anticholinesterase activity.

ACKNOWLEDGEMENTS

The authors are thankfull to Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), and Fundação de Amparo a Pesquisa do Estado de Goiás (FAPEG) for financial support and to Universidade Estadual de Goiás for technical support. LDD thanks CNPq for his PhD grant (232620/2014-8 GDE). GLBA is grateful for his postdoctoral grant Projeto "MATIS–Materiais e Tecnologias Sustentáveis – CENTRO-01-0145-FEDER-000014".

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