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MICROWAVE ASSISTED SOLVENT-FREE MANNICH BASES: SYNTHESIS, CHARACTERIZATION AND EFFECTS OF THESE COMPOUNDS ON hCA I AND hCA II ISOZYMES

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Mannich bases (**2a**–**d**) of aromatic amines were synthesized by using a conventional microwave technique under solvent-free conditions and characterized by IR and NMR (^{1}H and ^{13}C) and elemental analysis. The inhibitory effects of the synthesized Mannich bases were examined *in vitro* by using hydratase and esterase assays on carbonic anhydrase I and II isozymes (hCA, EC 4.2.1.1) purified from human erythrocyte cells. Acetazolamide was used as the control compound. The values of IC_{50}, the half-maximum inhibitory concentration, were found for hydratase and esterase activities. Only two compounds (**2b** and **2d**) exhibited poor hCA I and hCA II inhibition effects on esterase activity. In contrast, compounds **2a** and **2c** could be used as carbonic anhydrase activators as a result of the increased esterase activity of hCA I and hCA II isozymes.

Keywords: activator; carbonic anhydrase; glaucoma; isozyme

МАНИХОВИ БАЗИ ПОМОГНАТИ МИКРОБРАНОВО БЕЗ РАСТВОРУВАЧИ: СИНТЕЗА, КАРАКТЕРИЗАЦИЈА И ВЛИЈАНИЕ НА ОВИЕ СОЕДИНЕНИЈА ВРЗ ИЗОЕНЗИМИ НА hCA I И hCA II

Извршена е синтеза на Манихови бази (**2a**–**d**) со ароматични амини со употреба на вообичаени микробранови техники во отсутво на растворувачи, кои беа карактеризирани со IR и NMR (^{1}H и ^{13}C) и елементна анализа. Инхибиторните својства на синтетизираните Манихови бази беа испитани *in vitro* со употреба на тестови на хидратаза и естераза на изоензими на карбонската анхидраза I и II (hCA, EC 4.2.1.1) пречистени од хумани еритроцитни клетки. Како контролно соединение беше употребен ацетазоламид. Беа определени вредностите на IC_{50}, половина од максималната инхибиторна концентрација, за активностите на хидратаза и естераза. Само две соединенија (**2b** и **2d**) покажаа слабо инхибиторно дејство на hCA I и hCA II врз активноста на естераза. Од друга страна, соединенијата **2a** и **2c** можат да се користат како активатори на карбонска анхидраза како резултат на зголемена активност на естераза на изоензимите hCA I и hCA II.

Клучни зборови: активатор; карбонска анхидраза; глаукома; изоензим

1. INTRODUCTION

Carbonic anhydrase enzymes (CAs, EC 4.2.1.1) control pH in most tissues, including erythrocytes [1–3]. Recently, CAs have been extensively studied due to their potential applications in the prevention and treatment of a large number of diseases [4–6]. Sixteen isozymes have been described in the literature as members of the α-CA family [7–9]. Several of these isozymes (hCA I, hCA II and hCA IV) are found in human eyes and cause gradual loss of vision due to an elevation of intraocular

pressure (IOP), called glaucoma [10–13]. Since CA inhibitors lower the IOP, these compounds have been used for glaucoma treatment [14, 15].

Reactions involving amines and amino acid derivatives have been the most researched studies on the complexing of zinc, the active site of CAs [16–22]. The Mannich reaction involves condensation of ammonia, primary amines or secondary amines with formaldehyde and compounds containing at least one active hydrogen atom. This work would be a good example of the synthesis of Mannich compounds from amine derivatives by the Mannich reaction. Recently, chemists have been interested in reducing the environmental pollution caused by chemical solvents and developing eco-compatible synthetic methods [23–25]. Mannich bases have a wide range of pharmacological uses and exhibit important anti-fungal, antibacterial, anticancer [26, 27], analgesic, anti-inflammatory [28] and antituberculous properties [29].

We present an economical, environmentally friendly, solvent-free, one-step method for the reactions of four novel Mannich compounds (**2a**–**d**) using microwave irradiation (Fig. 1). Furthermore, we have investigated the inhibition effect of these compounds on human carbonic anhydrase (hCA I and hCA II) isozymes.

The structures of compounds **2a**–**d** were assigned by elemental analysis and IR and NMR (^{1}H and ^{13}C) spectroscopy. In addition, the inhibitory effects of **2a**–**d** were investigated, using acetazolamide (AAZ) as control compound.

2. EXPERIMENTAL

2.1. *Materials*

The reagents were used without purification. NMR spectra of the solution in $CDCl_3$ were recorded on a Bruker Avance DPX-300 instrument. A Leco CHNS-932 instrument was used for elemental analysis and IR studies were performed by Bruker Optics and Vertex 70 FT-IR. Reactions were carried out using a microwave oven manufactured by BEKO (1200 W, 2450 MHz).

2.2. *Synthesis of N-(2-fluorophenyl)-N-o-tolylmethanediamine (**2a**) and N-(4-fluorophenyl)-N-o-tolylmethanediamine (**2b**)*

Formaldehyde (4 mmol) was added dropwise to a solvent-free mixture of 2-methylphenylamine (4 mmol) and fluoro-substituted phenylamine (2-fluorophenylamine for **2a** and 4-fluorophenylamine for **2b**) (4 mmol) and the mixture irradiated by microwave at 200 W. Ethanol was used to wash the solid mixture and the residue was dried in air.

2a: Reaction time 45 min; (77 %); m.p. 153 ºC; ^{1}H NMR (300 MHz, $CdCl_3$) δ/ppm: 6.81–6.74 (m, 8H, ArH), 4.78 (s, 2H, N–CH_2), 2.21 (s, 3H, $ArCH_3$); ^{13}C NMR (300 MHz, $CdCl_3$) δ/ppm: 152.12, 140.21, 132,35, 129.36, 127.28, 126.14, 119.96, 117.37, 114.76 and 110.32 (aromatic carbons), 70.46 (CH_2), 13.87 (CH_3); IR (cm^{-1}): 3469, 3374 (NH), 3018 (Ar–CH), 2968, 2831 (aliphatic –CH), 1628, 1608, 1470, 1458 (C=C); Anal. Calcd. %, for $C_{14}H_{15}FN_2$ (M_r = 230.28): C 73.02, H 6.57, N 12.16; found: C 73.07, H 6.60, N 12.21.

2b: Reaction time 55 min; (72%); m.p. 142 ºC; ^{1}H NMR (300 MHz, $CdCl_3$) δ/ppm: 6.89–6.81 (m, 8H, ArH), 4.81 (s, 2H, N–CH_2), 2.22 (s, 3H, $ArCH_3$); ^{13}C NMR (300 MHz, $CdCl_3$) δ/ppm: 159.11, 148.23, 145.72, 128.76, 124.65, 119.32, 115.01, 108.45 (aromatic carbons), 69.21 (CH_2), 24.61 (CH_3); IR (cm^{-1}): 3375, 3270 (NH), 3070 (CH), 2933, 2882 (CH), 1638, 1603, 1502, 1451 (C=C); Anal. Calcd. %, for $C_{14}H_{15}FN_2$ (M_r = 230.28): C 73.02, H 6.57, N 12.16; found: C 73.05, H 6.61, N 12.23.

2.3. *Synthesis of N,N'-bis(2,3-dimethylphenyl)methanediamine (**2c**)*

Formaldehyde (4 mmol) was added dropwise to the 2,3-dimethylphenylamine (8 mmol). The solvent-free reaction mixture was irradiated by microwave at 200 W. Ethanol was used to wash the solid mixture and the residue dried in air.

2c: Reaction time 50 min; (82%); m.p. 138 ºC; ^{1}H NMR (300 MHz, $CdCl_3$) δ/ppm: 6.54–6.48 (m, 6H, ArH), 4.72 (s, 2H, N–CH_2), 2.74 (s, 6H, $ArCH_3$), 2.21 (s, 6H, CH_3); ^{13}C NMR (300 MHz, $CdCl_3$) δ/ppm: 144.92, 139.52, 128.79, 121.54, 119.85, 111.63 (aromatic carbons), 75.88 (CH_2), 21.74 (CH_3), 12.62 (CH_3); IR (cm^{-1}): 3435, 3375 (NH), 3069 (Ar–CH), 2932, 2879 (aliphatic –CH), 1646, 1603, 1504, 1435 (C=C); Anal. Calcd. %, for $C_{17}H_{22}N_2$ (M_r = 254.18): C 80.27, H 8.72, N 11.01; found: C 80.32, H 8.61, N 10.79.

2.4. *Synthesis of 2-((2-fluorobenzyl)aminomethyl)-4,5-dimethylphenol (**2d**)*

Formaldehyde (4 mmol) was added dropwise to a solvent-free mixture of 3,4-dimethylphenol (4 mmol) and (2-fluorophenyl)methanamine (4 mmol). The reaction mixture was irradiated by microwave at 200 W. Ethanol was used to wash the solid mixture and the residue dried in air.

2d: Reaction time 45 min; (70 %); m.p. 143 ºC; ^{1}H NMR (300 MHz, $CdCl_3$) δ/ppm: 7. 46 (s, 1H, OH), 7.29–6.69 (m, 6H, ArH), 5.31 (s, 2H, CH_2), 4.57 (s, 2H, N–CH_2), 2.24 (s, 3H, CH_3), 2.22 (s, 3H, CH_3); ^{13}C NMR (300 MHz, $CdCl_3$) δ/ppm: 156.83, 154.40, 151.96, 137.16, 136.40, 127.44, 124.56, 123.84, 123.76, 121.44, 117.77, 115.83 (aromatic carbons), 77.09 (CH_2), 76.78 (CH_2), 19.65.74 (CH_3), 18.90 (CH_3); IR (cm^{-1}): 3463 (O-H), 3372 (NH), 3023 (Ar–CH), 2937, 2836 (aliphatic –CH), 1612, 1577, 1493, 1451 (C=C); Anal. Calcd. %, for $C_{16}H_{18}FNO$ (M_r = 259.32): C 74.11, H 7.00, N 5.40; found: C 74.18, H 7.26, N 5.63.

2.5. *Purification of hCA I and hCA II isozymes*

The experimental procedure for the purification of hCA I and hCA II isozymes from erythrocytes has been described in detail in our previous studies [30–32]. Briefly, the erythrocyte hemolysate, adjusted to pH 8.7, was loaded onto an affinity column of Sepharose®4B-L-tyrosine-*p*-aminobenzene sulfonamide. After removal of unbound proteins and impurities, hCA I and hCA II were eluted using the following buffers: 1.0 M NaCl/25.0 mM Na_2HPO_4 (pH 6.3) and 0.1 M $NaCH_3COO$/0.5 M $NaClO_4$ (pH 5.6), respectively [33]. Quantification of the purified enzymes [34] and SDS-PAGE analysis [35] were performed.

2.6. *Examination of the inhibitory effects of hCA I and hCA II isozymes on the hydratase and esterase activity of the obtained compounds*

Solids (1 %), which are clinically used locally for the treatment of glaucoma [36], were prepared and the inhibitory effects on the hydratase and esterase activities of the hCA I and II isozymes investigated. Hydratase and esterase activity measurements were performed at five different concentrations of the appropriate inhibitor for hCA I and hCA II isozymes purified from human erythrocytes [30–32, 37–39]. The IC_{50} values were obtained *in vitro* for the synthesized compounds (**2a**–**d**), with AAZ as the control.

3. RESULTS AND DISCUSSION

In this work, four compounds (**2a**–**d**) were synthesized by the reaction of various aromatic amines with formaldehyde in the absence of solvent under microwave irradiation.

The newly synthesized Mannich bases **2a**–**d** are shown in Figure 1.

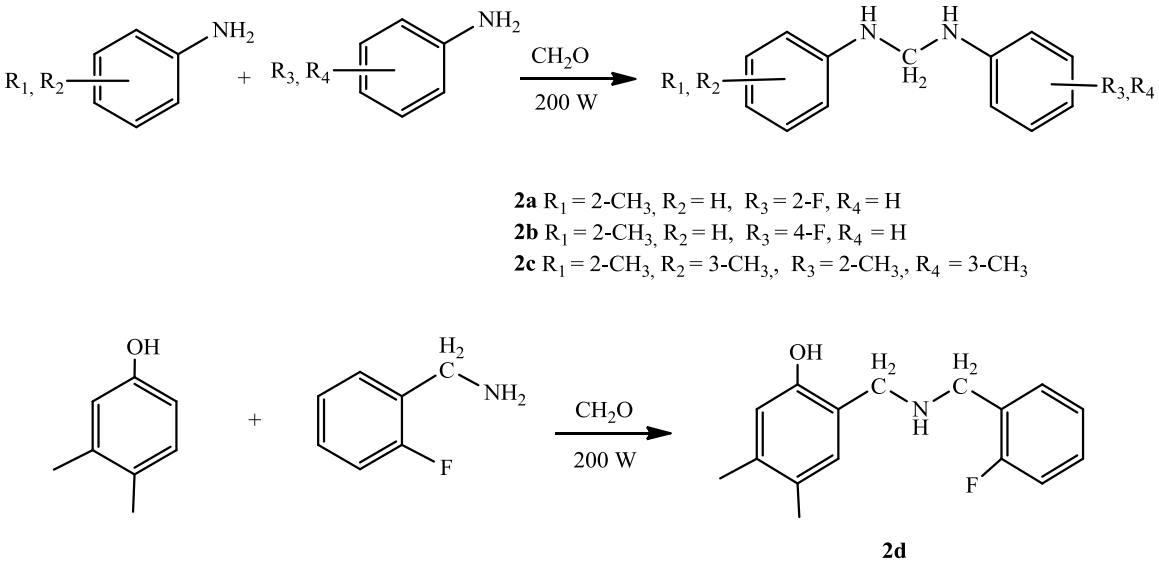

Fig.1. Syntheses of **2a**–**d**

3.1. *NMR (^{1}H and ^{13}C) spectra of compounds **2a**–**d**.*

NMR (^{1}H and ^{13}C) studies were carried out at room temperature in $CDCl_3$ solution (Figs. S1–4). The ^{1}H NMR spectra of **2a**–**d** displayed peaks in the aromatic region in the range 7.29–6.48 ppm. All compounds showed a singlet for methylene (CH_2) protons with an intensity of two hydrogens at 4.78, 4.81, 4.72 and 4.57 ppm, respectively. These singlets support the Mannich reaction between the amines and formaldehyde. A singlet with an intensity of three hydrogens for each CH_3 proton was observed at 2.21 and 2.22 ppm for compounds **2a** and **2b**, respectively.

Two singlets of six-proton intensity were found in the ^{1}H NMR spectrum of **2c** at 2.74 and 2.21 ppm, corresponding to the four methyl groups. Compound **2d** showed a singlet with an intensity of one hydrogen at 7.46 ppm for the OH proton. Also, three other singlets were observed for CH_2 and the two different CH_3 protons of compound **2d** at 5.31, 2.24 and 2.22 ppm, respectively. In addition, three singlets were observed in the NMR spectrum of compound **2d** at 5.31 ppm for CH_2 and at 2.24 and 2.22 ppm for the two different CH_3 protons.

Aromatic carbon atoms were observed in the range 159.11–108.45 ppm in the ^{13}C NMR spectra of **2a**–**d** (Figs. S1–4). Methylene (CH_2) carbon atoms confirming the Mannich reaction were observed at 70.46, 69.21, 75.88 and 76.78 ppm for the compounds **2a**–**d**, respectively. The carbon atom of the methyl (CH_3) groups in compounds **2a** and **2b** resonated at 13.87 and 24.61 ppm, respectively. Compound **2c** showed signals at 21.74 and 12.62 ppm for the two CH_3 carbon atoms. In the ^{13}C NMR spectrum of **2d**, a signal was observed at 77.09 ppm for the carbon atom of the benzyl CH_2 group.

3.2. *FT-IR measurements*

The IR spectra of all compounds (**2a**–**d**) displayed ν(N–H) stretching vibration bands in the range 3469–3270 cm^{-1} (Table 1). All compounds exhibited aromatic ν(C–H) bands in the range of 3070–3018 cm^{-1}. Aliphatic ν(C–H) bands for compounds **2a**–**d** were found in the range 2968–2932 and 2882–2831 cm^{-1}. A broad band was obtained at 3463 cm^{-1} consistent with the ν(O–H) stretching vibration in **2d**. All compounds exhibited stretching vibration in the range 1646–1435 cm^{-1} for C=C groups.

T a b l e 1

*Data of IR spectra (cm^{-1}) of **2a**–**d***

Assignment	ν(O-H)	ν(N-H)	ν(C-H)$_{ar.}$	ν(C-H)$_{aliph.}$	ν(C=C)
2a	–	3469(w)	3018(w)	2968(w)	1628(w) 1608(w)
		3374(w)		2831(w)	1470(m) 1458(s)
2b	–	3375(w)	3070(w)	2933(w)	1638(w) 1603(w)
		3270(w)		2882(w)	1502(s) 1451(w)
2c		3435(w)	3069(w)	2932(w)	1646(w) 1603(w)
		3375(w)		2879(m)	1504(s) 1435(w)
2d	3463(br)	3372(w)	3023(w)	2937(w)	1612(w) 1577(w)
				2836(w)	1493(s) 1451(w)

w: weak, br: broad, m: medium, s: strong

3.3. *In vitro inhibition studies*

After purification of hCA I and hCA II, the effects of the compounds on the hydratase and esterase activities of the isozymes were investigated *in vitro*. The synthesized compounds did not affect the hydratase activity of hCA I and hCA II. This indicates that **2a**–**d** did not interact with the zinc ion at the active site of the enzyme [40]. When the esterase activities of the isozymes were examined, an interesting situation arose. Compounds **2a** and **2c** were observed to enhance the esterase activity of hCA I and hCA II; however, **2b** and **2d** inhibited the esterase activities of these isozymes. The inhibitory effects on the esterase activity of **2b** (448.35 ± 2.01 and 392.38 ± 1.87 μM) and **2d** (291.60 ± 1.18 and 320.20 ± 1.18 μM) were considerably weaker than that of AAZ (0.43 ± 0.05 and 0.32 ± 0.08 μM), which was the control compound (Table 2). However, this study is important as it adds to the literature new CA activators (**2a** and **2c**) and inhibitors (**2b** and **2d**). Also, the effects on isozymes of the compounds studied in this work will shed light on other works in this area.

T a b l e 2

*Esterase IC_{50} values (μM) of synthesized compounds (**2a**–**d**)*

Compound	hCA I[b,c]	hCA II[b,c]
AAZ[a]	0.43 ± 0.05	0.32 ± 0.08
2a	Activated	Activated
2b	448.35 ± 2.01	392.38 ± 1.87
2c	Activated	Activated
2d	291.60 ± 1.18	320.20 ± 1.18

[a]AAZ was used as reference compound
[b]Mean ± standard error, from three different assays
[c]$p < 0.0001$ for all analyses

4. CONCLUSIONS

In this work, four new compounds (**2a**–**d**) were prepared in the absence of solvent under microwave conditions. This one-step method was quite clean, efficient, economical and practical. The structures of the compounds obtained were clarified by FT-IR and NMR (^{1}H and ^{13}C) spectroscopy and elemental analysis. All the results were found to be in harmony with the suggested structures (Fig.1). The inhibitory effects of the synthesized compounds on hCA I and hCA II isozymes were assessed by hydratase and esterase activity assays, and IC_{50} values were also determined. The synthesized compounds did not show inhibitory effects on the hydratase activity of hCA I and hCA II. Only compounds **2b** and **2d** were determined to have a weak inhibitory effect on the esterase activity of hCA I and hCA II. However, it was observed that compounds **2a** and **2c** increased the esterase activity of hCA I and hCA II isozymes. For this reason, these compounds (**2a** and **2c**) may be good CA activators.

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