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RESEARCH ARTICLE

Synthesis and characterization of phenolic Mannich bases and effects of these compounds on human carbonic anhydrase isozymes I and II

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Abstract
Mannich bases **2a-f** derived from 3,4-dimethylphenol (**1**), formaldehyde and different amines are prepared and subjected to spectral (IR, ^{1}H and ^{13}C NMR) and elemental analyses. The inhibition of two human carbonic anhydrase (hCA, EC 4.2.1.1) isozymes I and II, with **1** and synthesized Mannich bases **2a-f** and acetazolamide (**AAZ**) as a control compound was investigated *in vitro* by using the hydratase and esterase assays. In relation to hydratase and esterase activities of the half maximal inhibitory concentration (IC_{50}) and the inhibition equilibrium constants (K_i)values were determined. Only two compounds (**2a** and **2e)**exhibit weak hCA II inhibitory effects on esterase activity. IC_{50} and *K*i values for **2a** and **2e** with respect to esterase activity of hCA II are0.88 × 10^3 and 6.3–7.6 µM and 0.44 × 10^3 and 19.0–96.4 µM,respectively. On the contrary, compounds **2b** and **2d** might be used as CA activators due to increasing esterase activity of hCA I and hCA II isozymes.

Keywords: Mannich base, 3,4-dimethylphenol, NMR, carbonic anhydrase, hydratase and esterase activities

Introduction

Carbonic anhydrase (CA, EC 4.2.1.1) has a catalytic function of CO_2 hydration. In the active site of CA, Zn(II) ion has a tetrahedral geometry and is coordinated by the three histidine imidazoles and a water molecule[1]. At least 16 CA isozymes were described up to now in mammals, the most active ones as catalysts for carbon dioxide hydration being CA II and CA IX[2–5]. Several of these isozymes (hCA II and hCA IV) are present in human eyes[6–9] causing glaucoma, which is a group of diseases characterized by a gradual loss of the visual field due to an elevation in intraocular pressure (IOP), and being the second leading cause of blindness worldwide[9,10]. Since CA inhibitors have been shown to reduce IOP exclusively by lowering the aqueous humour flow and these compounds have been used for the treatment of glaucoma for years[11,12]. The rate-determining step for the CO_2 hydration reaction catalyzed by CAs is the proton transfer reaction from the water bound to the Zn(II) ion to the reaction medium, with generation of the zinc hydroxide species of the enzyme[13–20].

Several anions behave as inhibitors for the CO_2 hydration reaction[21,22] and coordinate directly to Zn(II) ion. A number of studies on the zinc model complexes as the active site of CA, which consist of amines and amino acid derivatives are the most investigated ones[23–31]. The interaction of all 16 mammalian CA isozymes with several types of phenols, some of which are widely used as antioxidant food additives or as drugs has been also investigated[32–35]. Some antioxidant phenol derivatives have been showed effective hCA II inhibitory effects, in the same range as the clinically used sulfonamide acetazolamide[36]. This has given impetus for the synthesis of Mannich bases from these compounds using Mannich reaction. Mannich reaction offers a convenient method for introduction of the basic aminoalkyl chain, which alters the biological profile and physicochemical

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characteristics[37]. Various drugs obtained from Mannich reaction have proved more effective and less toxic than their parent antibiotics[38]. In the present study, previously synthesized **2a**[39] and novel Mannich bases **2b-f** have been prepared by using microwave assist reaction of 3,4-dimethylphenol (**1**) with formaldehyde and various amines (Scheme 1) which contain both functional phenolic–OH group and -NHR or -NR_2 groups. They have been characterized by spectral (IR, ^{1}H and ^{13}C NMR) and elemental analyses. Furthermore, we have purified human CA I and II (hCA I and hCA II) from erythrocytes and examined the *in vitro* inhibition effects of synthesized compounds together starting compound (**1**) and acetazolamide (**AAZ**) as a control compound.

Materials and methods

All reagents were of the highest grade, commercially available and used without further purification. ^{1}H and ^{13}C NMR spectra were recorded with DPX-300 MHz Bruker Avance NMR spectrometer (Bio spin Gmbh, Germany). Elemental analyses for C, H and N were performed on a Leco CHNS-932 instrument. IR spectra were recorded on a Bruker Optics, vertex 70 FT-IR spectrometer using ATR techniques. A domestic microwave oven manufactured by BEKO was used for the microwave-assisted reactions at highest power (1200 W) and 2450 MHz operating frequency.

The general experimental procedure for 2a-2f

To mixture of 3,4-dimethylphenol (**1**) (0.1 mol) and amine compound (0.1 mol), formaldehyde (0.1 mol) was added dropwise with stirring then irradiated by microwave. After cooling to room temperature the residue was washed with ethanol and dried in air.

2-((dimethylamino)methyl)-4,5-dimethylphenol (2a)

Reaction time: 15 min; (white solid, 90%); mp 70 ˚C; ^{1}H-NMR (d_6-DMSO, ppm):8.77 (s, 1H, H^1), 6.77 (s, 1H, H^3), 6.51 (s, 1H, H^8), 3.46 (s, 2H, H^{10}), 2.58 (s, 3H, H^7), 2.48 (s, 3H, H^5), 2.10 (s, 3H, CH_3), 2.07 (s, 3H, CH_3); ^{13}C-NMR (d_6-DMSO, ppm): 154.20 (C^2), 138.64 (C^4), 131.86 (C^8), 128.32 (C^6), 122.43 (C^3), 119.81 (C^9), 59.99 (C^{10}), 47.21 (CH_3), 18.96 (C^5), 18.82 (C^7); IR (cm^{-1}): 3492 (OH), 3012 (C-H)$_{ar.}$, 2980, 2800 (C-H)$_{aliph.}$, 1600-1457 (Ar C=C), 820 (ring); Anal. Calcd for $C_{11}H_{17}NO$: C, 73.70; H, 9.56; N, 7.81. Found: C, 73.86; H, 9.15; N, 7.93.

2-((diethylamino)methyl)-4,5-dimethylphenol (2b)

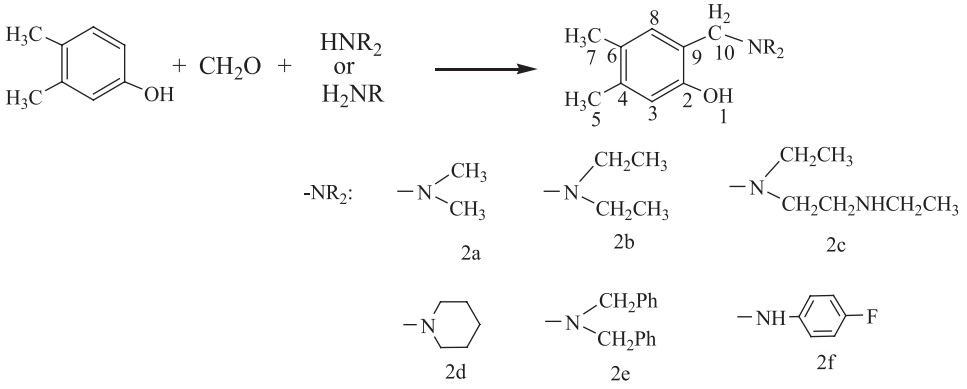
Reaction time: 35 min; (brown oil, 89%); ^{1}H-NMR (d_6-DMSO, ppm):9.56 (s, 1H, H^1), 6.65 (s, 1H, H^3), 6.44 (s, 1H, H^8), 3.50 (s, 2H, H^{10}), 2.42 (q, J = 7.03 Hz 4H, CH_2), 2.20 (s, 3H, H^7), 2.10 (s, 3H, H^5), 0.92 (t, J = 7.01 Hz 6H, CH_3); ^{13}C-NMR (d_6-DMSO, ppm): 149,53 (C^2), 136.72 (C^4), 129.43 (C^8), 125.50 (C^6), 119.52 (C^3), 116.67 (C^9), 55.56 (C^{10}), 45.15 (CH_2), 19.02 (C^5), 18.47 (C^7), 10.97 (CH_3); IR (cm^{-1}): 3485 (OH), 3006 (C-H)$_{ar.}$, 2961, 2873 (C-H)$_{aliph.}$, 1600-1440 (Ar C=C), 750 (ring); Anal. Calcd for $C_{13}H_{21}NO$: C, 75.32; H, 10.21; N, 6.76. Found: C, 75.34; H, 10.26; N, 6.81.

2-((ethyl(2-(ethylamino)ethyl)amino)methyl)-4,5-dimethylphenol (2c)

Reaction time: 60 min; (white solid, 85%); mp 120˚C;^{1}H-NMR (d_6-DMSO, ppm):10.19 (s, 1H, H^1), 6.80 (s, 1H, H^3), 6.49 (s, 1H, H^8), 3.57 (s, 2H, H^{10}), 2.05 (s, 3H, H^7), 2.09 (s, 3H, H^5), 3.34 (s, 1H, NH), 2.58(t, J = 4.71 Hz 3H, CH_3), 2.50 (q, J = 4.71 Hz 2H, CH_2), 2.49 (t, J = 4.68 Hz 2H, CH_2), 2.48 (m, 2H, CH^2), 0.99-0.89 (m, 5H, C_2H_5); ^{13}C-NMR (d_6-DMSO, ppm): 151.32 (C^2), 141.09 (C^4), 131.11 (C^8), 128.42 (C^6), 119.99 (C^3), 119.87 (C^9), 57.79 (C^{10}), 20.01 (C^5), 19.99 (C^7), 55.02 (NCH_2CH_2), 48.67 (NCH_2CH_3), 46.23 (NCH_2CH_2NH), 44.3 ($NHCH_2CH_3$), 15.68 ($NHCH_2CH_3$), 13.42 (NCH_2CH_3); IR (cm^{-1}): 3490 (OH), 2977 (NH), 3011 (C-H)$_{ar.}$, 2968, 2831 (C-H)$_{aliph.}$, 1628-1458 (Ar C=C), 748 (ring); Anal. Calcd for $C_{15}H_{26}NO$: C, 71.95; H, 10.47; N, 11.19. Found: C, 71.94; H, 10.09; N, 11.45.

4,5-dimethyl-2-(morpholinomethyl)phenol (2d)

Reaction time: 30 min; (brown oil, 92%);^{1}H-NMR (d_6-DMSO, ppm):9.82 (s, 1H, H^1), 6.82 (s, 1H, H^3), 6.55 (s, 1H, H^8), 3.58 (s, 2H, H^{10}), 3.59 (t, J = 4.03 Hz 4H, OCH_2), 2.40 (t, J = 4.03 Hz 4H, NCH_2), 2.12 (s, 3H, H^7), 2.11 (s, 3H, H^5); ^{13}C-NMR (d_6-DMSO, ppm): 154.89 (C^2), 136.29 (C^4), 130.93 (C^8), 126.33 (C^6), 119.34 (C^3), 117.80 (C^9), 66.71 (OCH_2), 59.26 (C^{10}), 53.17 (NCH_2), 19.63 (C^5), 18.79 (C^7). IR (cm^{-1});



Scheme 1. Syntheses of **2a-f**.

3489 (OH), 3010 (C-H)$_{ar.}$, 2990, 2907 (C-H)$_{aliph.}$, 1635-1448 (Ar C=C), 750 (ring); Anal. Calcd for $C_{13}H_{19}NO_2$: C, 70.56; H, 8.65; N, 6.33. Found: C, 70.62; H, 8.66; N, 6.37.

2-((dibenzylamino)methyl)-4,5-dimethylphenol (2e)

Reaction time: 35 min; (white solid, 96%); mp 96 ˚C; ^{1}H-NMR (d_6-DMSO, ppm):10.41 (s, 1H, H^1), 7.28-7.87 (m, 10H, C_6H_5), 6.67 (s, 1H, H^3), 6.66 (s, 1H, H^8), 3.69 (s, 2H, H^{10}), 3.62 (s, 4H, CH_2), 2.20 (s, 3H, H^7), 2.17 (s, 3H, H^5); ^{13}C-NMR (d_6-DMSO, ppm): 153.62 (C^2), 137.92 (C^4), 137.46–126.98 (C_6H_5-*C*H), 132.94 (C^8), 129.85 (C^6), 119.85 (C^3), 119.74 (C^9), 63.56 (C_6H_5-*C*), 55.84 (C^{10}), 19.20 (C^5), 18.80 (C^7); IR (cm^{-1}): 3495 (OH), 3015 (C-H)$_{ar.}$, 2950, 2850 (C-H)$_{aliph.}$, 1560-1455 (Ar C=C), 736 (ring); Anal. Calcd for $C_{23}H_{25}NO$: C, 83.34; H, 7.60; N, 4.23. Found: C, 83.36; H, 7.41; N, 4.35.

2-((4-fluorophenylamino)methyl)-4,5-dimethylphenol (2f)

Reaction time: 20 min; (white solid, 82%); mp 145˚C;^{1}H-NMR (d_6-DMSO, ppm):9.13 (s, 1H, H^1), 6.90 (s, 1H, H^3), 6.92 (s, 1H, H^8), 6.50 (m, 2H, C_6H_4), 6.40 (m, 2H, C_6H_4), 5.82 (s, 1H, NH), 4.06 (s, 2H, H^{10}), 2.08 (s, 3H, H^7), 2.04 (s, 3H, H^5); ^{13}C-NMR (d_6-DMSO, ppm): 156.68 (C_6H_4-*C*), 151.02 (C^2), 145.01 (C_6H_4-*C*), 138.52 (C^4), 130.00 (C^8), 129.01 (C^6), 126.13 (C^3), 120.01 (C^9), 119.13 (C_6H_4-*C*H), 53.42 (C^{10}), 116.52 (C_6H_4-*C*H), 21.01 (C^5), 19.68 (C^7); IR (cm^{-1}): 3488 (OH), 3276 (NH), 3007 (C-H)$_{ar.}$, 2976, 2884 (C-H)$_{aliph.}$, 1502-1453 (Ar C=C), 763 (ring); Anal. Calcd for $C_{15}H_{16}FNO$: C, 73.45; H, 6.57; N, 5.71. Found: C, 73.36; H, 6.37; N, 5.85.

Purification of isoenzymes hCA-I and hCA-II from human erythrocytes

In order to purify hCA-I and hCA-II isoenzymes, first, human blood was centrifuged at 1500 rpm for 20 min, and after the removal of the plasma, the erythrocytes were washed with an isotonic solution (0.9% NaCl). After that, the erythrocytes were lysed with 1.5 volume of ice-cold water. The lysate was centrifuged at 20,000 rpm for 30 min to remove cell membranes and non-lysed cells. The pH of the supernatant was adjusted to 8.7 with tris and was then loaded onto an affinity column containing Sepharose-4B-L-tyrosine-p-aminobenzene sulfonamide as the binding group. After extensive washing with 25 mM tris–HCl/22 mM Na_2SO_4 (pH 8.7), the hCA-I and hCA-II isoenzymes were eluted with 1.0 M NaCl/25 mM Na_2HPO_4 (pH 6.3) and 0.1 M CH_3COONa/0.5 M $NaClO_4$ (pH 5.6)[40,41]. The amount of purified protein was estimated by the Bradford method[42] and SDS–PAGE was carried out to determine whether the elute containing the enzyme[43].

Hydratase activity assay

Carbonic anhydrase activity was assayed by following the hydration of CO_2 according to the method described by Wilbur and Anderson[44]. CO_2-hydratase activity as an enzyme unit (EU) was calculated by using the equation $((t_0\text{-}tc)/tc)$ where t_0 and tc are the times for pH change of the nonenzymatic and the enzymatic reactions, respectively. IC_{50} values (the concentration of inhibitor producing a 50% inhibition of CA activity) have been obtained as *in vitro* for the synthesized compounds **2a**-**f**, **1** and **AAZ** as the control compound.

Esterase activity assay

Carbonic anhydrase activity was assayed by following the change in absorbance at 348 nm of 4-nitrophenylacetate (NPA) to 4-nitrophenylate ion over a period of 3 min at 25°C using a spectrophotometer (CHEBIOS UV–VIS) according to the method described in the literature[45,46]. The enzymatic reaction, in a total volume of 3.0 mL, contained 1.4 mL of 0.05 M tris–SO_4 buffer (pH 7.4), 1 mL of 3 mM 4-nitrophenylacetate, 0.5 mL H_2O and 0.1 mL enzyme solution. A reference measurement was obtained by preparing the same cuvette without enzyme solution. IC_{50} values have been obtained as *in vitro* for free the synthesized compounds **2a**-**f**, **1** and **AAZ** as the control compound.

Determination of K_i values

The method for determination of *K*i values is described elsewhere[47–51]. In the first part of this study, IC_{50} values have been obtained as *in vitro*. IC_{50} of the inhibitors (the synthesized compounds **2a**-**f**, **1** and **AAZ** as the control compound) were assayed by the hydrolysis of *p*-nitrophenylacetate on esterase activities of CA isoenzymes in the presence of various inhibitor concentrations. The absorbance was determined at 348 nm after 3 min[47]. Regression analysis graphs were drawn by plotting inhibitor concentrations versus enzyme activity by using Microsoft Excel Program.

In the second part of the study, enzyme activity was measured in the presence of five different substrate concentrations at each of these inhibitor concentrations (30%, 50%, and 70%), and the data were linearized with Lineweaver–Burk plot in order to obtain K_i values.

Result and discussion

We herein report the syntheses of title compounds **2a-f** by treatment of 3,4-dimethylphenol (**1**), with various amines in the presence of formaldehyde. The reactions were carried out under microwave (MW) irradiation and solvent free condition. The one-step Mannich reaction of **2a-f** is given in Scheme 1.

^{1}H-NMR and ^{13}C-NMR spectra of 2a-f

The ^{1}H and ^{13}C-NMR spectra of compounds **2a-f** were recorded in d_6-DMSO solution at room temperature using TMS as internal standard. In the ^{1}H NMR spectra the signals of the respective protons of the prepared compounds **2a-f** were verified on the basis of their chemical shifts, multiplicities and coupling constants. The NMR spectra of **2a-f** exhibited a broad singlet in the

range 10.41-8.77 ppm which correspond to protons H^1 (O-H)[40]. The chemical shift of the aromatic protons of the benzene ring are observed in the range 6.90-6.65 ppm for H^3 protons and 6.92-6.44 ppm for H^8 protons. The ^{1}H NMR spectra of **2a-f** showed a singlet with 2 H intensity each, in the range 4.06-3.46 ppm assigned to the H^{10} (CH_2) protons, confirming the Mannich condensation of 3,4-dimetylphenol, formaldehyde and amines[52,53]. In the ^{1}H NMR spectra of compounds **2a-f** showed two singlets with 3 H intensity each, in the range 2.58-2.05 and 2.48-2.04 ppm for protons H^7 and H^5, respectively.

^{13}C NMR spectra of **2a-f** exhibited peaks in the range 154.89–149.53, 141.09-136.29, 132.94–129.43, 129.85–125.50, 126.13–119.34 and 120.01-116.67 ppm due to C^2, C^4, C^8, C^6, C^3 and C^9 carbon atoms, respectively. The carbon atoms (C^{10}) for **2a-f** were observed in the range 59.99-53.42 ppm. The signals in the range 21.01-18.96 and 19.99-18.47 are assigned to C^5 and C^7 carbon atoms, respectively.

FTIR measurements

The IR spectra of all compounds showed vibrational bands in the range 3495-3485 cm^{-1} for O-H group. The bands at 3276 and 2977 cm^{-1} are assigned to vibrations associated with the N-H moiety for compounds **2f** and **2c**, respectively[54]. Aromatic C-H stretching vibrations for all compounds are observed in the range 3015-3006 cm^{-1}. The bands in the range 2990-2950 and 2907-2800 cm^{-1} are attributable to aliphatic C-H stretching vibrations for compounds **2a-f**. The IR spectra of all compounds showed vibrational bands in the range 1635-1440 cm^{-1} for C=C group[55]. The bands of ring are located in the range 820-736 cm^{-1}.

In vitro inhibition studies

Inhibition effects on hCA I and hCA II isozymes of the synthesized compounds (**2a-f**) and acetazolamide (**AAZ**) as the control compound were studied by hydratase and esterase activity methods. The related *K*i values were also determined for each compound in order to compare with inhibition effects of starting compound (**1**) and **AAZ** (Table 1).

According to *in vitro* studies, there is no inhibition effects observed for **1** and synthesized compounds (**2a**-**f**) on hydratase activities of hCA I and hCA II isozymes, and for **2c** and **2f** on esterase activities of hCA I and hCA II isozymes. Starting compound **1** showed esterase activities on hCA I and hCA II. However, IC_{50} values for the esterase activities of **1** (1.03×10^3 μM for hCA I and 0.93×10^3 μM for hCA II, respectively) were greater than the IC_{50} values of **AAZ** (5.9 μM for hCA I and 4.3 μM for hCA II, respectively). In relation to esterase activities, the inhibition equilibrium constants (*K*i) were also determined. Compound **1** exhibited *K*i values as 8.5-79.6 μM for hCA I and 15.3-67.3 μM for hCA II, respectively indicating poorer inhibition effects compared to **AAZ** (5.0-6.1 μM for hCA I and 3.5-4.1 μM for hCA II, respectively).

Compounds **2a** and **2e** showed inhibitory effects on esterase activity of hCA II, nevertheless they did not showed any inhibitory effects of hCA I isozyme. The esterase IC_{50} and the *K*i values for **2a** and **2e** (0.88×10^3 and 6.3–7.6 μM for hCA II, respectively and 0.44×10^3 and 19.0-96.4 μM for hCA II, respectively) are higher than the esterase IC_{50} and the *K*i values of AAZ (4.3 and 3.5-4.1 μM for hCA II, respectively). Similar to **1**, these compounds are also weaker inhibitors than **AAZ**. No inhibition effects were observed for compounds **2b** and **2d** on hCA I and hCA II isozymes but these compounds increased the esterase activities of these isozymes. Therefore, **2b** and **2d** compounds might be evaluated as carbonic anhydrase activators.

The compounds used in this study have both functional phenolic -OH group and -NHR or -NR_2 groups together in a molecule which have not reinforced their CA (I and II) inhibitory effects. This might be due to acid-base reaction between them, although they are good CA inhibitors separately[23–28,30,32,36].

Conclusion

An efficient, clean, economic, and one-pot procedure for the synthesis of Mannich bases (**2a-f**) has been used by the three-component coupling of aldehyde, amine, and 3,4-dimethylphenol (**1**) under microwave irradiation, solvent free conditions, short reaction time with excellent yields of the products. All of the synthesized compounds **2a-f** have been completely characterized by IR, ^{1}H and ^{13}C NMR and elemental analyses and all results are consistent

Table 1. IC_{50} and K_i values forsynthesized compounds (**2a-f**), **1** and **AAZ** withhCA I and hCA II isozymes.

	Esterase IC_{50} (μM)		K_i values (μM)	
Inhibitor	hCA I	hCA II	hCA I	hCA II
AAZ	5.9	4.3	5.0–6.1	3.5–4.1
1	1.03×10^3	0.93×10^3	18.5–79.6	15.3–67.3
2a	No inhibition	0.88×10^3	No inhibition	6.3–7.6
2b	Activated	Activated	No inhibition	No inhibition
2c	No inhibition	No inhibition	No inhibition	No inhibition
2d	Activated	Activated	No inhibition	No inhibition
2e	No inhibition	0.44×10^3	No inhibition	19.0–96.4
2f	No inhibition	No inhibition	No inhibition	No inhibition

with the proposed structures (Scheme 1). Inhibition effects of synthesized compounds (**2a-f**)on hCA I and hCA II isozymes were studied by hydratase and esterase activity methods and then *K*i values were determined. None of the synthesized compounds (**2a-f**) did not show any inhibition effect on hydratase activity of hCA I and hCA II. Only **2a** and **2e** showed weak inhibitory effects on esterase activity of hCA II. On the contrary, compounds **2b** and **2d** might be good CA activators due to increasing esterase activity of hCA I and hCA II isozymes.

Declaration of interest

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