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# Synthesis of New Schiff Bases and Assessment of Their in vitro Biological Effects on Acetylcholinesterase and Carbonic Anhydrase Isoenzymes Activities

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**Abstract**—In this study, three new Schiff bases have been synthesized by the reactions of commercially available phenylglycinol, phenylalaninol, and leuicinol with 4-{[2-(4-formylphenoxy)ethyl](methyl)amin}benzaldehyde and characterized by $^{1}H$ and $^{13}C$ NMR, FTIR, and UV-Vis spectroscopy and LCMS/MS. In vitro effects of synthesized new Schiff bases on human erythrocyte carbonic anhydrase I (hCA I) and II (hCA II) isoenzymes and acetylcholinesterase (AChE) activity were investigated. Schiff base synthesized from phenylglycinol showed no meaningful effect on hCAI and hCAII. Schiff bases synthesized from phenylalaninol and leuicinol exhibited a strong activation effect on hCAI and hCAII. On the other hand, all of the synthesized three Schiff bases exhibited a strong inhibitory effect on AChE activity.

**Keywords:** Schiff bases, carbonic anhydrase, acetylcholinesterase

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Alzheimer's disease (AD) emerging due to neuron and synapse losses in various parts of the central nervous system is a progressive and deadly neurodegenerative disease [1]. This disease particularly affects the elderly population and is the most common cause of dementia. Today, there are 50 million people affected by dementia, and this number is foreseen to reach 152 million by 2050 [2, 3]. The AD pathology is not completely understood, and there is no cure for it; therefore, effective drug development studies are extremely important for the treatment of AD [4].

Acetylcholinesterase (AChE; E.C.3.1.1.7) is an important metabolic enzyme that catalyzes the hydrolysis of acetylcholine to acetic acid and choline [5–7]. AD is caused by a low acetylcholine level in brain [8]. Therefore, AD is treated with AChE inhibitors [9]. For this reason, the discovery of new AChE inhibitors is important for the development of drugs against the AD disease.

It is also known that carbonic anhydrase enzyme levels in the brain of Alzheimer's patients are significantly reduced [10, 11]. Carbonic anhydrases (CAs, EC 4.2.1.1), an important class of metalloenzymes, catalyze the conversion of carbon dioxide to bicarbonate and proton, and play an essential role in signal processing, long-term synaptic transformation, and attentional gating of memory storage. They have seven different genetic families (α-, β-, γ-, δ-, ζ-, η-, and θ-CAs) and 16 isoenzymes [6, 12], Carbonic anhydrase dysfunction is associated with mental retardation, Alzheimer's disease, and aging [13]. Today, CA inhibitors are used in the treatment of many diseases from glaucoma to epilepsy [6, 12–14], but CA activators have not yet introduced in clinical practice [11, 14]. Recent studies showed that CA activators, such as phenylalanine and imidazole, can provide a significant pharmacological improvement in synaptic activity, mechanical learning, and memory enhancement, which is associated with aging and AD [11].

Schiff bases have a wide range of biological activities, including anti-inflammatory, antimicrobial, anticancer, antioxidant, antimalarial, antifungal, antiviral [29], analgesic, anticonvulsant, antituberculosis, and an-

thelmintic [30]. Some Schiff bases were found to exhibit an inhibitory effect on acetylcholinesterase [31]. Biological activity and enzyme inhibition studies of compounds containing Schiff bases and amino alcohol moieties have been published in recent years.

Considering the importance of AChE inhibitors and CA activators in the treatment of Alzheimer's disease and in view of the recent interest to the enzyme inhibition activity of Schiff bases derived from amino alcohols [32, 33], in the present work we synthesized and characterized new Schiff bases and assessed their in vitro effects on the hCA I and hCA II isoenzymes and acetylcholinesterase enzyme.

## RESULTS AND DISCUSSION

The protocol of the synthesis of the previously unknown Schiff bases **4a–4c** by the reactions of phenylglycinol (**3a**), phenylalaninol (**3b**), and leuicinol (**3c**) with 4-{[2-(4-formylphenoxy)ethyl](methyl)-amin}benzaldehyde (**2**) and the structures of the synthesized compounds are presented in the Scheme 1. Compound **2** gives two aldehyde proton peaks at 9.89 and 9.79 ppm in the $^{1}$H NMR spectrum and two aldehyde carbon peaks at 190.29 and 190.71 ppm in the $^{13}$C NMR spectrum. This indicated that the structure contains two unsymmetrical aldehyde groups. The LCMS spectra of the compound **2** molecular ion peak at the expected *m/z* values. The structures of Schiff bases **4a–4c** were proved by spectral methods. The IR spectra of all the compounds contain the C=N and OH stretching bands at 1637–1642 and 3218–3248 cm$^{-1}$, respectively, as well as aromatic CH stretching bands at 2938–2954 cm$^{-1}$. The LCMS spectra of the Schiff bases contain molecular ion peaks at the expected *m/z* values.

When $^{1}$H NMR spectra of Schiff bases are examined the most important peak to be considered is the proton peak in HC=N group. This structural feature was observed in the case of Schiff bases. Two different imine peaks were observed between δ 8.20 and 8.30 ppm in all three Schiff bases. Amino alcohol groups in the proton peak of OH group were observed to be between δ 4.24 and 4.3313 ppm. The aromatic and aliphatic peaks were consistent with the structure.

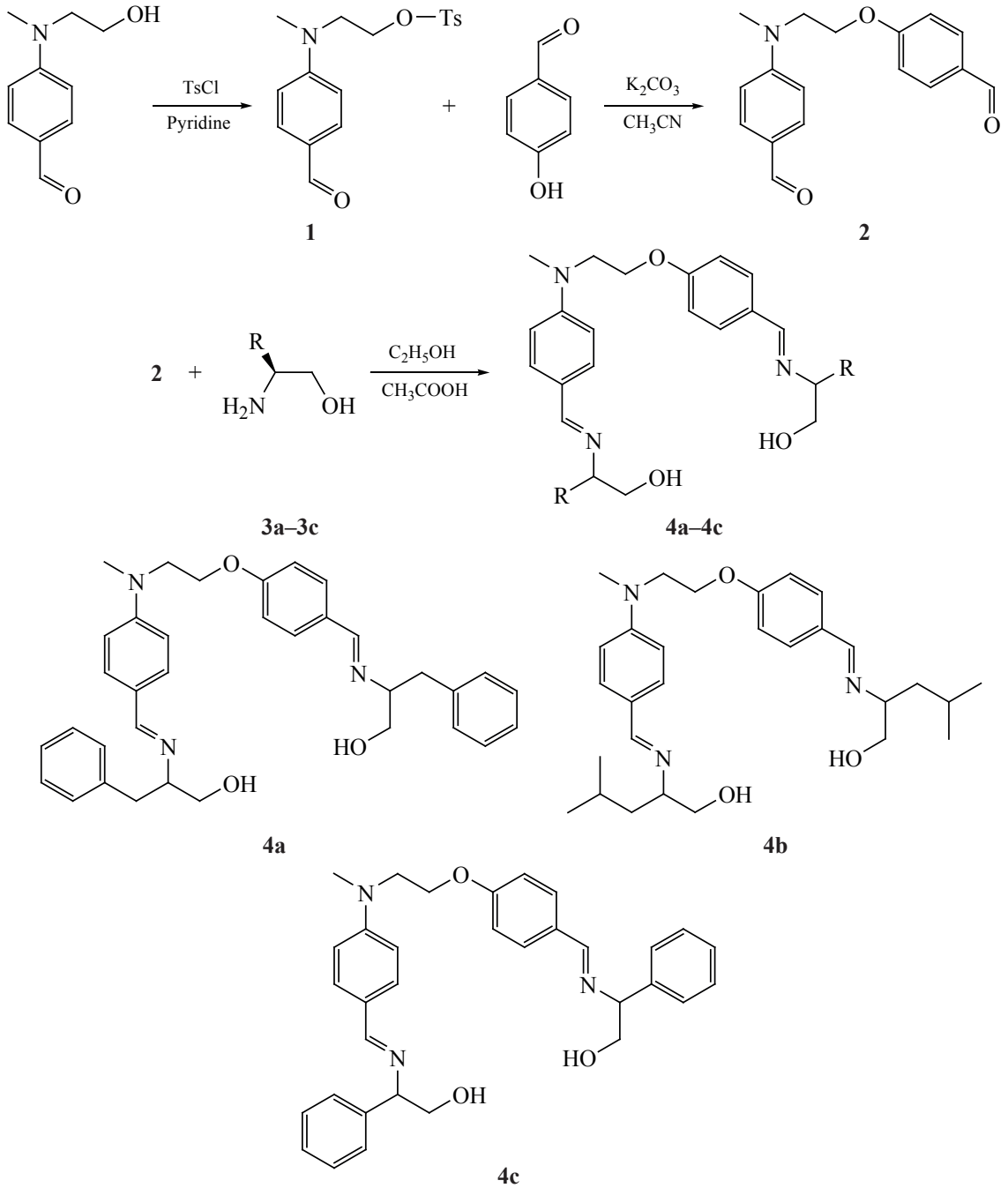
In this study, in vitro effects of the synthesized Schiff bases on the hCA I, hCA II and AChE activities were assessed. For this purpose, the hCA I and hCA II isoenzymes were first of all purified from human erythrocytes by CNBr-activated Sepharose-4B-L-tyrosine sulfanilamide affinity chromatography. The purification results are presented in Table 1. As seen from the Table 1, yields of pure hCA I and hCA II were, respectively, 64.4 and 44.4%, purification factors 1360 and 1715, and specific activities 1208 and 1523 EU/mg. Activity measurements of hCA I and hCA II isoenzymes were made by the method, described in [21]. It was found that compounds **4b** and **4c** showed strong activation effects on the hCA I and hCA II isoenzymes at very low concentrations, while compound **4a** did not work both as an inhibitor or an activator. The % activities of compounds **4b** and **4c** were plotted versus their concentrations (Figs. 1 and 2), and these plots were used to determine the activation constants ($K_A$): 168.41 (**4b**) and 87.36 (**4c**) μM for hCA I and 9.05 (**4b**) and 3.72 (**4c**) μM for hCA II. Considering that a lower $K_A$ value points to a higher binding affinity, we can conclude that Schiff base **4c** exhibits the strongest activation effect on hCA II.

The in vitro effects of the synthesized Schiff bases of AChE enzyme activity were assessed by the Ellman's method [22]. It was found that Schiff bases **4a–4c** all had an inhibition effect on AChE activity. The half-maximal inhibitory concentrations ($IC_{50}$) and inhibition constants ($K_i$) of the products were measured. The $IC_{50}$ values of **4a**, **4b**, and **4c** were estimated at 2.14, 2.16, and 33.3 μM, respectively. As seen, compounds **4a** and **4b** are much stronger AChE inhibitors than **4c**. Accordingly, the $K_i$ constants, which relate to the binding affinity of the inhibitor to enzyme [6], for compounds **4a** and **4b** are lower compared to **4c**: 0.439 ± 0.084, 0.708 ± 0.171, and 15.23 ± 1.807 μM, respectively. According to results, all the Schiff bases demonstrated a competitive inhibition mechanism, and **4a** showed the strongest binding affinity to AChE (Fig. 2).

## EXPERIMENTAL

All reagents and solvents, chemical materials for enzyme studies, and AChE (CAS no. 9000-81-1) were purchased from Sigma-Aldrich and used without further purification. Mass spectra were recorded with Thermo Scientific TSQ Quantum Access Max LC-MS/MS spectrometers in the ethyl acetate mixture. The IR spectra were recorded on a Nicolet-6700 ATR-FT-IR spectrophotometer in the range 4000–400 cm$^{-1}$ in KBr pellets. The $^{1}$H and $^{13}$C NMR spectra were measured on a Bruker Ultrashield spectrometer at 400 and 75 MHz, respectively, in $CD_3Cl$ solutions at room

**Scheme 1.**




R = Bn (**a**), isobutyl (**b**), Ph (**c**).

temperature. The UV–Vis spectra were recorded on a Thermo Scientific Genesis 10S spectrophotometer. The melting points were determined on a Thermo Scientific Electrothermal IA9100 apparatus.

Compound **1** was synthesized by the procedure in [15].

**Synthesis of 4-{*N*-[2-(4-formylphenoxy)ethyl]-*N*-methylamino}benzaldehyde (2).** 4-Hydroxybenzaldehyde (0.61 g, 5 mmol) and $K_2CO_3$ (4.14 g, 30 mmol) were added to a solution of 4-[*N*-methyl-*N*-(2-{[(4-methylphenyl)sulfonyloxy]ethyl}amino)benzaldehyde (1.33 g, 4 mmol) in acetonitrile (30 mL). The mixture was stirred in an oil bath at 82°C for 48 h with TLC monitor-

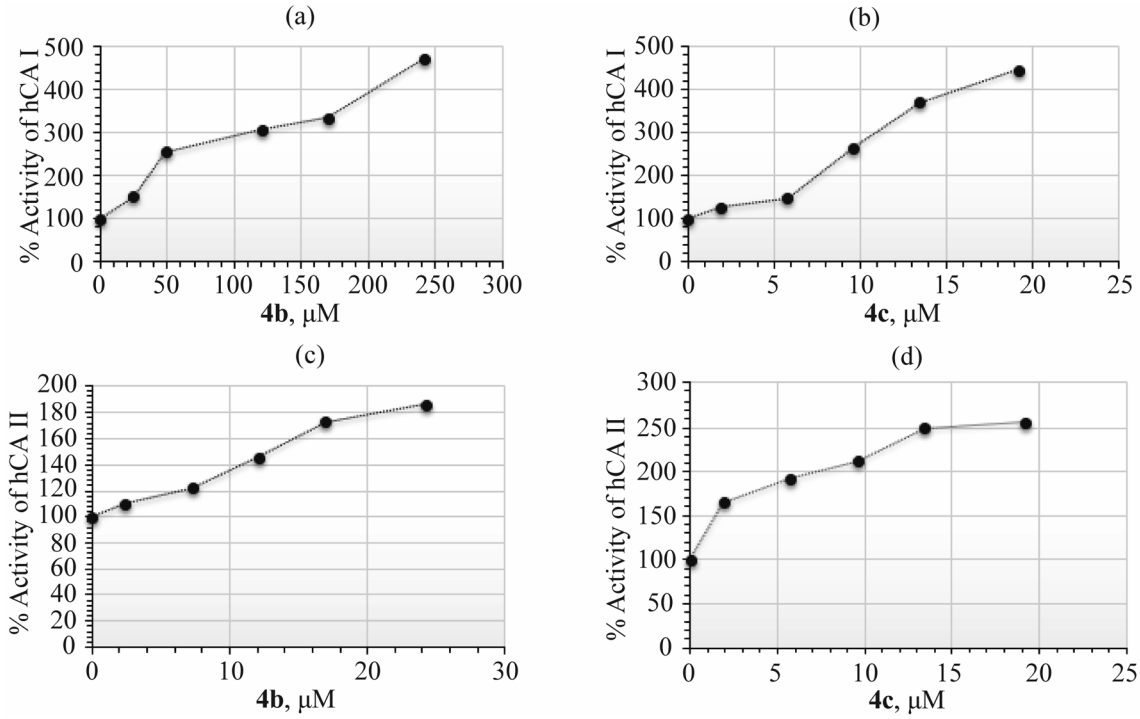



**Fig. 1.** Activation plots for (a), (b) hCA I and (c), (d) hCA II. Shiff base: (a), (c) **4b** and (b), (d) **4c**.

ing. At the end of the reaction, the mixture was filtered, evaporated, and diluted with water. The aqueous organic phase was extracted with ethyl acetate (3 × 10 mL), and the organic phase was then washed in triplicate with 1 N sodium hydroxide, dried with $Na_2SO_4$ and evaporated to leave a yellow solid. Yield 93%, mp 110–111°C. $^1$H NMR spectrum, δ, ppm: 3.20 d (3H, *J* 0.9 Hz), 3.88–3.99 m (2H), 4.26–4.29 m (2H), 6.77–6.86 m (2H), 6.96–7.06 m (2H), 7.72–7.80 m (2H), 7.86 d (2H, *J* 8.7 Hz), 9.77 s (1H), 9.89 s (1H). $^{13}$C NMR spectrum, δ, ppm: 39.49, 51.38, 65.59, 111.16, 114.71, 125.79, 130.33, 132.02, 132.12, 153.16, 163.35, 190.29, 190.71. Mass spectrum (LC-MS), *m/z*: 284.07 [*M* + H]. $M_{calc}$ 283.33.

**Synthesis of Schiff bases 4a–4c** (*general procedure*). Compound **2** (1 mmol) and amino alcohol (2 mmol) were dissolved in ethyl alcohol (30 mL), and three drops acetic acid was added. The mixture was stirred in an oil bath at 78°C for 16 hours. At the end of the reaction, the mixture was evaporated, and the solid residue was recrystallized from ethyl acetate.

**2-[(*E*)-4-{*N*-[2-(4-{(*E*)-[(1-Hydroxy-3-phenylpropan-2-yl)iminomethyl]phenoxy}ethyl)-*N*-methylamino]benzylidene}amino]-3-phenylpropan-1-ol (4a).** Yield 82%, mp 92–93°C. IR spectrum, ν, cm$^{-1}$: 3247.30 (OH), 2953.21 (CH), 1636.54 (C=N), 1430.68 (ring C–C). $^1$H NMR spectrum, δ, ppm: 1.21–1.32 m (1H), 2.07 d (1H, *J* 1.0 Hz), 2.81–2.91 m (1H), 2.97 d (3H, *J* 13.6 Hz), 3.10–3.26 m (1H), 6.88 d (2H, *J* 8.5 Hz), 3.50 s (1H), 3.77–3.85 m (1H), 3.87–3.94 m (1H), 4.13–4.24 m (1H), 6.73 d (2H, *J* 8.5 Hz), 7.13–7.22 m (1H), 7.26–7.30 m (1H), 7.59 d.d (2H, *J* 11.3, 8.8 Hz), 7.92 d (2H, *J* 16.5 Hz), 8.23 s (1H), 8.27 s (1H). $^{13}$C NMR spectrum, δ, ppm: 39.38, 40.71, 64.78, 64.92, 65.54, 66.42, 111.18, 115.30, 124.42, 125.41, 126.19, 127.38, 128.00, 129.54, 129.97, 136.27, 150.18, 161.42, 161.78. Mass spectrum (LC-MS), *m/z*: 550.19 [*M* + H]. $M_{calc}$ 549.70.

**2-[(*E*)-4-{*N*-[2-(4-{(*E*)-[(1-Hydroxy-3-phenylpropan-2-yl)iminomethyl]phenoxy}ethyl)-*N*-methylamino]benzylidene}amino]-4-methylpentan-1-ol (4b).** Yield 93%, mp 120–121°C. IR spectrum, ν, cm$^{-1}$: 3248.90 (OH), 2954.23 (CH), 1637.31 (C=N), 1431.55 (ring C–C). $^1$H NMR spectrum, δ, ppm: 0.83–1.01 m (3H), 1.27–1.38 m (1H), 1.57 d (2H, *J* 14.7 Hz), 1.90 s (1H), 3.13 d (3H, *J* 4.3 Hz), 3.33–3.45 m (1H), 3.64–3.75 m (2H), 3.85 t (1H, *J* 5.7 Hz), 4.20 t (1H, *J* 5.7 Hz), 6.71–6.81 m (2H), 6.84–6.94 m (2H), 7.26–7.30 m (1H), 7.66 d.d (2H, *J* 12.2, 11.9 Hz), 8.20 s (1H), 8.25 s (1H). $^{13}$C NMR spectrum, δ, ppm: 21.75, 23.52, 24.36, 39.31, 41.12, 51.56, 65.36, 66.64, 70.65, 111.46, 114.40, 124.35,

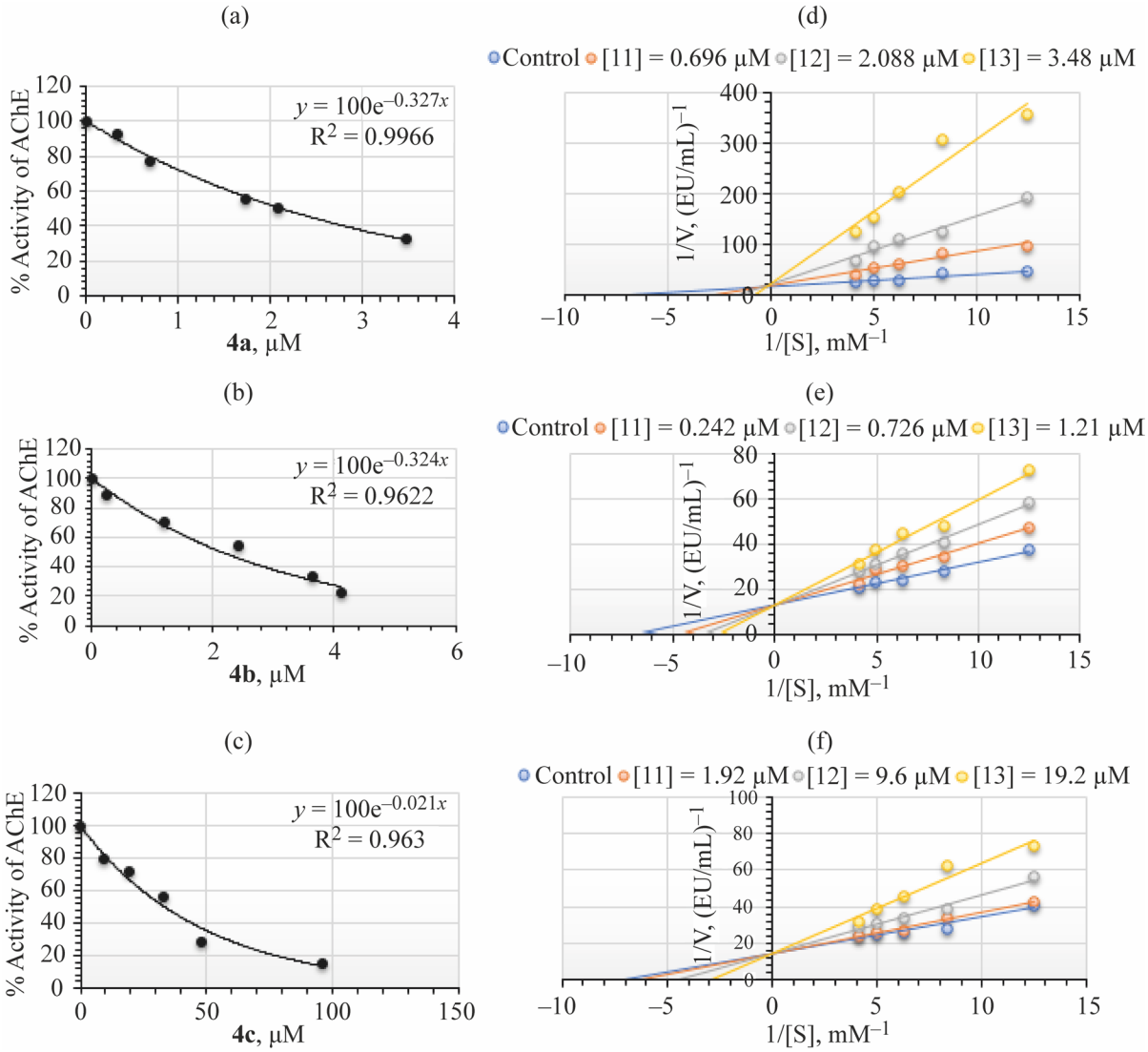



**Fig. 2.** (a), (b), (c): $IC_{50}$ graphs of **4a**, **4b**, **4c** for AChE, respectively; (d), (e), (f): $K_i$ graphs of **4a**, **4b**, **4c** for AChE, respectively.

129.16, 129.95, 150.66, 160.64, 161.00, 161.64. Mass spectrum (LC-MS), *m/z*: 482.23 [*M* + H]. $M_{calc}$ 481.67.

**2-[(*E*)-4-{*N*-[2-(4-{(*E*)-[(1-Hydroxy-3-phenylpropan-2-yl)iminomethyl]phenoxy}ethyl)-*N*-methylamino]benzylidene}amino]-2-phenylethan-1-ol (4c).** Yield 92%, mp 100–101°C. IR spectrum, ν, $cm^{-1}$: 3218.92 (OH), 2938.14 (CH), 1642.50 (C=N), 1420.57 (ring C–C). $^1H$ NMR spectrum, δ, ppm: 3.13–3.15 m (1H), 3.20 d (3H, *J* 5.4 Hz), 3.81–4.03 m (1H), 4.20–4.24 m

**Table 1.** Purification results of hCA I and hCA II isoenzymes isolated from human erythrocytes

| Purification Steps | Activity, EU/mL | Total volume, mL | Protein, mg/mL | Total Protein, mg/mL | Total Activity, EU | Specific Activity, EU/mg | Yield, % | Purification factor, fold |
|---|---|---|---|---|---|---|---|---|
| Erythrocyte Hemolysate | 320 | 45 | 360 | 16200 | 14400 | 0.888 | 100 | 1.00 |
| hCAI | 580 | 16 | 0.480 | 7.68 | 9280 | 1208 | 64.4 | 1360 |
| hCAII | 640 | 10 | 0.42 | 4.20 | 6400 | 1523 | 44.4 | 1715 |

(1H), 4.45–4.49 m (2H), 6.72–6.82 m (2H), 687–6.94 m (2H), 7.30–7.33 m (1H), 7.35–7.40 m (2H), 7.42–7.50 m (2H), 7.65–7.81 m (2H), 8.24 s (1H), 8.30 s (1H). $^{13}$C NMR spectrum, δ, ppm: 39.30, 39.49, 51.17, 64.89, 67.75, 111.16, 111.45, 114.14, 127.38, 128.56, 130.18, 132.72, 133.02, 141.14, 151.14, 160.52, 162.07, 162.69. Mass spectrum (LC-MS), *m/z*: 522.14 [*M* + H]. $M_{calc}$ 521.65.

**Purification of hCA I and hCA II.** A fresh human erythrocyte suspension was obtained from the University Hospital Blood Center of Erzurum Ataturk University. A 30-mL portion of the erythrocyte suspension was hemolyzed with 1.5 volumes of an ice-cold distilled water. The hemolysate was centrifuged at 20000 rpm and 4°C for 30 min to remove ghost and intact cells. After centrifugation, the precipitate was discarded, and the erythrocyte hemolysate (45 mL) in the upper phase was taken for use in purification processes [12, 16–18].

A Sepharose 4B-L-tyrosine-sulfanylamide affinity column was equilibrated with 25 mM Tris-HCl/0.1 M $Na_2SO_4$ buffer (pH 8.7). The erythrocyte hemolysate (45 mL) was adjusted to pH 8.7 with solid Tris and then loaded onto the column. After extraction, the column was washed with 25 mM Tris-HCl/22 mM $Na_2SO_4$ buffer (pH 8.7) to remove impurities. The hCA I isoenzyme was eluted first with 1.0 M NaCl/25 mM $Na_2HPO_4$ buffer (pH 6.3), and then the hCA II isoenzyme was eluted with 0.1 M $NaCH_3COO$/0.5 M $NaClO_4$ buffer (pH 5.6). During the purification process, absorbance was measured at 280 nm in all eluates to monitor hCA I and hCA II elution, and the Wilbur–Anderson method was used to measure the activity of hCA I and hCA II in the eluates. The collected active hCA I and hCA II fractions were dialyzed against 50 mM Tris-$SO_4$ (pH 7.4) overnight. After dialysis, the isoenzymes were stored at –80°C for use in kinetic studies in small fractions of 1 mL [12, 16–18]. The Bradford protein assay was used for protein quantification in crude hemolysates and during enzyme purification steps [19]. Additionally, SDS-PAGE was used for purity control of the isoenzymes after purification [20].

**Biological activity studies.** The activities of hCA I and hCA II were measured by the method in [21], based on the conversion of 4-nitrophenyl acetate to 4-nitrophenolate within 3 min at 25°C and subsequent spectrophotometric absorbance measurements at 348 nm. The isoenzyme activities were measured at five different concentrations of Schiff bases **4a–4c** (Concentrations of **4a** for hCA I and hCA II: 0.348, 0.696, 1.74, 2.088, 3.48 μM. Concentrations of **4b** for hCA I: 24.2, 48.4, 121, 169.4, 242.0 μM. Concentrations of **4c** for hCA I: 1.92, 5.76, 9.6, 13.44, 19.2 μM. Concentrations of **4b** for hCA II: 2.42, 7.26, 12.1, 16.94, 24.2 μM. Concentrations of **4c** for hCA II: 1.92, 5.76, 9.6, 13.44, 19.2 μM). Plots of % activity vs. Schiff base concentration were constructed (Fig. 1), and the activation constants $K_A$ were calculated by SigmaPlot 12 for Windows (Systat Software, San Jose, CA).

The AChE activity was measured by Ellman assay [22], based on the quantification of the absorbance of 5-thio-2-nitrobenzoic acid, which is equivalent to the amount of thiochline formed by the AChE-catalyzed hydrolysis of acetylthiocholine. Acetylthiocholine iodide was used as the substrate. Absorbance measurements were performed at 412 nm [6, 22] at five different concentrations of Schiff bases **4a–4c** (Concentrations of **4a** for AChE: 0.348, 0.696, 1.74, 2.088, 3.48 μM. Concentrations of **4b** for AChE: 0.242, 1.21, 2.42, 3.63, 4.114 μM. Concentrations of **4c** for AChE: 9.6, 19.2, 32.64, 48, 96 μM). The control activity was considered as 100%. Plots of % AChE activity vs. Schiff base concentration were constructed and used to determine the $IC_{50}$ value each compound (Fig. 2). The $K_i$ values and types of inhibition were determined from the Lineweaver–Burk plots [23] constructed with the activities measured at five different substrate concentrations (0.08, 0.12, 0.16, 0.20, 0.24 mM) for three different Schiff base concentrations (Concentrations of **4a** for AChE: 0.696, 2.088, 3.48 μM. Concentrations of **4b** for AChE: 0.242, 0.726, 1.21 μM. Concentrations of **4c** for AChE: 1.92, 9.6, 19.2 μM).

## CONCLUSIONS

In conclusion, three new Schiff bases **4a**, **4b**, and **4c** were synthesized and characterized. Compounds **4b** and **4c** showed strong activation effects at very low concentrations on the hCA I and hCA II isoenzymes, while compound **4a** did not show any effect. All the three Schiff bases showed strong inhibition effect on AChE, but the strongest inhibition effect was observed with compound **4a**. The results of the present work may be useful for the development of new CA activators and AChE inhibitors for the treatment of various diseases, especially AD.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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