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Article

Preparation of Two Maleic Acid Sulfonamide Salts and Their Copper(II) Complexes and Antiglaucoma Activity Studies

Cengiz Yenikaya,*,[a] Halil İlkimen,[a] Mehmet Melih Demirel,[a] Burçin Ceyhan,[a] Metin Bülbül[b] and Ekrem Tunca[b]

[a]Department of Chemistry and [b]Department of Biochemistry, Faculty of Arts and Sciences, Dumlupınar University, 43100 Kütahya, Turkey

Two novel proton transfer compounds $(HAP)^+(SAMAL)^-$ and $(HBI)^+(SAMAL)^-.H_2O$ were obtained from (*E*)-4-oxo-4-(4-sulfamoylphenylamino)but-2-enoic acid (HSAMAL) and 2-aminopyridine (AP) or 1*H*-benzimidazole (BI), respectively. Copper(II) complexes of salts and of HSAMAL have also been prepared. They have been characterized by elemental, spectral, thermal analyses, magnetic measurement and molar conductivity. Human carbonic anhydrase isozymes (hCA I and hCA II) were purified from erythrocytes by using affinity chromatography as 84.40 and 188.71 fold, respectively. The inhibitory effects of synthesized compounds and acetazolamide (AAZ, control compound) on the hydratase and esterase activities of hCA isozymes have been studied as *in vitro* to find out their antiglaucoma potentials. The inhibition constant (K_i) values of the compounds were in the range of 0.18 ± 0.007 to 10.24 ± 0.014 μmol L^{-1} for hCA I, and 0.12 ± 0.004 to 130.11 ± 0.021 μmol L^{-1} for hCA II.

Keywords: (*E*)-4-oxo-4-(4-sulfamoylphenylamino)but-2-enoic acid, 2-aminopyridine, 1*H*-benzimidazole, proton transfer, carbonic anhydrase inhibition

Introduction

The sulfonamides constitute an important class of drugs, with several types of pharmacological agents possessing antibacterial, antiglaucoma, anti-inflammatory, protease inhibitors and antitumor activity, among others.[1-3] Carbonic anhydrase (CA) is ubiquitous metalloenzyme that catalyze the two physiologically important processes: the first, the hydration of carbon dioxide to bicarbonate ion and a proton, and the second, the dehydration of bicarbonate ion.[4-6] These simple reactions provide acid-base equilibrium and ion equilibrium, so carbonic anhydrases are involved in many physiological processes such as pH regulation, ion transport, electrolyte secretion, calcification, and some biosynthetic reactions.[4,7] Among the mammalian carbonic anhydrases, α-carbonic anhydrases, sixteen different CA isoforms are expressed in humans.[5,6] Human carbonic anhydrase (hCA) II isozyme which is present on the human eye[8] interacts with some membrane transport proteins, including chloride/bicarbonate exchanger, sodium/proton exchanger, and the sodium bicarbonate cotransporter.[9] Thus hCA II plays a key role in the production of aqueous humor.[8] As a result, elevation in intraocular pressure (IOP) causes gradual loss of visual field called as glaucoma.[10-12] One of the methods used in glaucoma treatment is to reduce the production of aqueous humor[10,13] by using carbonic anhydrase inhibitors.[4,5,11,13,14]

2-Aminopyridines and benzimidazoles serve as useful chelating ligands in a variety of inorganic and organometallic applications,[15-17] and also act as monodentate ligands which coordinate the metal ions through the nitrogen of the ring[16-21] in most cases. In addition, there are several works on 2-aminopyridine complexes in which the amino group also participates in coordination.[22,23]

The chemistry of copper(II) carboxylate complexes with N-donor ligands especially with AP (2-animopyridine, **5**) and BI (1*H*-benzimidazole, **8**) has been extensively studied over the past few decades.[16,18-21] Two types of ligands, generally acids (e.g., carboxylic acids) and bases, e.g., AP or BI, are brought together before coordination to the metal ion in order to prepare the mixed ligand complex compounds.[18,24-26] The metal complexes with mixed ligands have shown better biological activities than the simple ones.[18,19,27,28]

In this study, two novel proton transfer compounds $(HAP)^+(SAMAL)^-$ (**6**) and $(HBI)^+(SAMAL)^-.H_2O$ (**9**),

*e-mail: cengiz.yenikaya@dpu.edu.tr

obtained from the acid **3** [HSAMAL, (*E*)-4-oxo-4-(4-sulfamoylphenylamino)but-2-enoic acid][29] and the bases **5** or **8** (1*H*-benzimidazole), respectively, had their copper(II) complexes **7** ($C_{20}H_{22}N_6O_6SCu$), **10** ($C_{24}H_{28}N_6O_{10}SCu_2$) and **4** ($C_{20}H_{22}N_4O_{12}S_2Cu$) prepared and characterized by nuclear magnetic resonance (NMR) of ^1H and ^{13}C, Fourier transform infrared (FTIR), ultraviolet-visible (UV-Vis) spectroscopy, elemental and thermal analyses, magnetic measurement and molar conductivity.

Experimental

Materials

All reagents were of the highest grade commercially available and used without further purification. Elemental analyses for C, H, N and S were performed on Elementar Vario III EL (Hanau, Germany) and Cu was detected with PerkinElmer Optima 4300 DV ICP-OES (Wellesley, USA). ^1H NMR spectra were recorded with Bruker DPX FT NMR (500 MHz) spectrometer (Karlsruhe, Germany) with $SiMe_4$ as internal standard and 85% H_3PO_4 as an external standard. FTIR spectra were recorded in the 4000-400 cm^{-1} region with Bruker Optics, Vertex 70 FTIR spectrometer (Ettlingen, Germany) using ATR techniques. Thermal analyses were performed on SII Exstar 6000 TG/DTA 6300 model (Shimadzu Corp., Kyoto, Japan) using platinum crucible with 10 mg sample. Measurements were taken in the static air within a 30-900 ºC temperature range. The UV-Vis spectra were obtained for aqueous solutions of the compounds (10^3 M) with a Shimadzu UV-2550 spectrometer (Kyoto, Japan) in the range of 200-900 nm. Magnetic susceptibility measurements at room temperature were performed using a Sherwood Scientific Magway MSB MK1 (Cambridge, UK) model magnetic balance by the Gouy method using $Hg[Co(SCN)_4]$ as calibrant. The molar conductances of the compounds were determined in water/ethanol (1:1, v/v) and DMSO (10^{-3} mol L^{-1}) at room temperature using a WTW Cond 315i/SET Model conductivity meter (Weilheim, Germany).

Synthesis of proton transfer salts (**6** and **9**) and metal complexes (**4**, **7** and **10**)

The starting compound **3**, the acid, was synthesized according to the literature.[29] ^1H NMR and ^{13}C NMR of the compound are given in Table 1 and other spectroscopic data can be found in Tables S1 and S2, Figures S1 and S2 at Supplementary Information.

A solution of **3** (1.351 g, 5 mmol) in 25 mL ethanol was added to 5 mmol amine solution of 5 (0.471 g) for **6**; or **8** (0.591 g) for **9** in 25 mL ethanol. The mixture was refluxed for 3 h and then was cooled to room temperature to give white solid of **6** or **9** (1.640 g, 90% yield for **6**, 1.554 g, 80% yield for **9**).

A solution of 1 mmol $Cu(CH_3COO)_2.H_2O$ (0.199 g) in water (10 mL) was added drop wise to the solution of 1 mmol of **3** (0.270 g) for **4**; or **6** (0.364 g) for **7**; or **5** (0.388 g) for 10 in water/ethanol (1:1, v/v) (20 mL) with stirring for four days at room temperature to give blue amorphous solid for **4** (0.239 g, 75% yield); or green amorphous solid for **7** (0.269 g, 50% yield); or red amorphous solid for **10** (0.607 g, 75% yield).

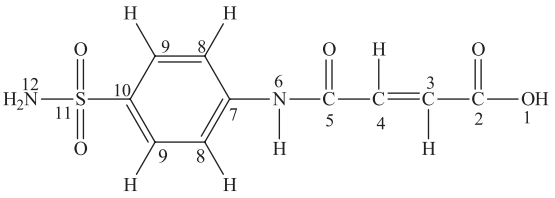
Anal. calcd. for **4** ($C_{20}H_{22}N_4O_{12}S_2Cu$): C 37.65, H 3.48, N 8.78, S 10.05, Cu 9.96%; found: C 37.70, H 3.43, N 8.79, S 10.15, Cu 9.95%; for **6** ($C_{15}H_{16}N_4O_5S$): C 49.44, H 4.43, N, 15.38, S 8.80%; found: C 49.40, H 4.45, N 15.40, S 8.85%; for **7** ($C_{20}H_{22}N_6O_6SCu$): C 44.65, H 4.12, N 15.62, S 5.96, Cu 11.81%; found: C 44.65, H 4.15, N 15.65, S 5.90, Cu 11.60%; for **9** ($C_{17}H_{16}N_4O_5S$): C 52.57, H 4.15, N 14.43, S 8.26%; found: C 52.57, H 4.15, N 14.45, S 8.30%; for **10** ($C_{24}H_{28}N_6O_{10}SCu_2$): C 40.05, H 3.92, N 11.68, S 4.46, Cu 17.66%; found: C 39.90, H 3.95, N 11.65, S 4.40, Cu 17.20%.

In addition, simple metal complexes of **5** (**12**, $[Cu_2(Ac)_4(AP)_2]$)[30] and **8** (**13**, $[Cu_2(Ac)_4(BI)_2]$)[31] were synthesized according to the literature in order to compare the inhibition studies with **4**, **6**, **7**, **9** and **10**.

Purification of carbonic anhydrase I and II isoenzymes from human erythrocytes

Erythrocytes were purified from human blood. The blood samples were centrifuged at 1500 rpm for 20 min and plasma was removed. Later, red cells were washed with isotonic solution (0.9% NaCl), and the erythrocytes were hemolyzed with 1.5 volumes of ice-cold water. Cell membranes were removed by centrifugation at 4 °C, 20000 rpm for 30 min. The pH of hemolysate was adjusted to 8.7 with solid TRIS (tris(hydroxymethyl)aminomethane). The hemolysate was applied to affinity column (Sepharose®4B-L-tyrosine-*p*-aminobenzene sulfonamide) pre-equilibrated with 25.0 mM TRIS-HCl/0.1M Na_2SO_4 (pH 8.7). After extensive washing with a solution of 25.0 mM TRIS-HCl/22.0 mM Na_2SO_4 (pH 8.7), the hCA I and hCA II isoenzymes were eluted with the solution of 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.[32] For quantitative protein determination, the Bradford method was used with bovine serum albumin as a standard.[33] Also purity control of the isoenzymes was performed with SDS-PAGE after the purification.[34]

Table 1. ^{1}H NMR and ^{13}C NMR chemical shifts (δ) with coupling constants and assignments for compound **3**



H_1	δ 12.9 (1H, s)	C^2	δ 168
H_3	δ 6.50 (1H, d, $^3J_{H3\text{-}H4}$ 11.96 Hz)	C^3	δ 137
H_4	δ 6.36 (1H, d, $^3J_{H4\text{-}H3}$ 11.95 Hz)	C^4	δ 119
H_6	δ 10.8 (1H, s)	C^5	δ 164
H_8, H_9	δ 7.80 (4H, s)	C^7	δ 139
H_{12}	δ 7.30 (2H, s)	C^8	δ 132
		C^9	δ 131
		C^{10}	δ 142

Determination of hydratase and esterase activities of hCA I and hCA II

The CO_2 hydratase activity of the purified enzyme was determined at 0 °C in a veronal buffer (pH 8.15) with the pH-stat method as the indicator and saturated carbon dioxide solution as the substrate in a final volume of 4.2 mL. The time (in seconds) taken for the solution to change from pH 8.15 to pH 6.50 was measured. The enzyme unit (EU) is the enzyme amount that reduces the non-enzymatic reaction time by 50%. The activity of an enzyme unit was calculated by using the equation $(t_0\text{-}t_c)/t_c$, where t_0 and t_c are times for pH change of the non-enzymatic and enzymatic reactions, respectively.[35]

Esterase activity was assayed by following the change in the absorbance at 348 nm of 4-nitrophenylacetate to 4-nitrophenylate ion over a period of 3 min at 25 °C using a spectrophotometer according to the method described in the literature.[36] 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), 1.0 mL of 3.0 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.

Determination of IC_{50} and K_i values of the compounds

To determine the IC_{50} values (the concentration of inhibitor producing a 50% inhibition of CA activity) of the simple ligands (4-aminobenzenesulfonamide (**1**), maleic anhydride (**2**), **5** and **8**), proton transfer salt (**6** and **9**), metal complexes (**4**, **7** and **10**), and acetazolamide (AAZ, **11**), as the control compound, hydratase and esterase activities of CA isoenzymes were assayed in the presence of various inhibitor concentrations as mentioned above. Regression analysis graphs were drawn by plotting the percent enzyme activity *vs.* inhibitor concentration and IC_{50} values were calculated.[24,26]

To determine inhibition constant (K_i) values as well as the inhibition type, three different inhibitor concentrations giving 30, 50 and 70% inhibition were selected. At each of these inhibitor concentrations, enzyme activity was measured in the presence of various substrate concentrations (0.3, 0.4, 0.5, 0.6 and 0.7 mM) and the data were linearized with Lineweaver-Burk plot for ν_{max} and the K_i determination. Enzyme activity was also measured in the presence of the same substrate concentrations but in the absence of any inhibitor to determine the ν_{max}.[24,26]

Statistical analysis

All the presented data were confirmed in at least three independent experiments and were expressed as the mean ± standard deviation (SD). Data were analyzed by using a one-way analysis of variance for multiple comparisons (SPSS 13.0, SPSS Inc., Chicago, IL). $p < 0.0001$ was considered to be statistically significant.

Results and Discussion

NMR studies of $(HAP)^+(SAMAL)^-$ (**6**) and $(HBI)^+(SAMAL)^-.H_2O$ (**9**)

The ^{1}H NMR and ^{13}C NMR spectra of the compounds for **6** and **9** were obtained in DMSO-d_6 with and without D_2O at room temperature using TMS (tetramethylsilane)

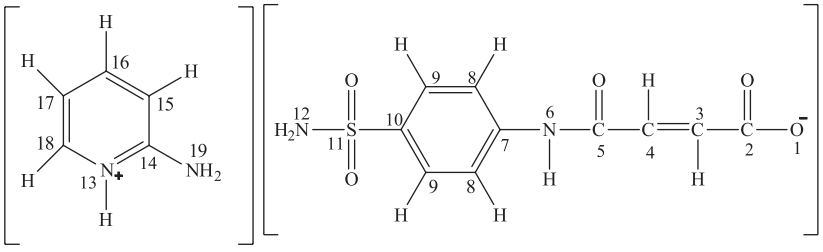
as internal standard (Figures S4-S7 at Supplementary Information). The ^{1}H signals were assigned on the basis of chemical shifts, multiplicities, intensities of the signals and coupling constants. Tables 2 and 3 lists complete ^{1}H NMR and ^{13}C NMR spectra of the compounds **6** and **9**.

In ^{1}H NMR spectrum of **6** (Figure S4a, Table 2), the H^3 and H^4 protons of the **3** are doublets with 1H intensities and they are observed at δ 6.36 (H^3, $^3J_{H3\text{-}H4}$ 12.14 Hz) and 6.33 (H^4, $^3J_{H4\text{-}H3}$ 12.11 Hz, indicating trans position of H^3 and H^4). The symmetrical H^8 and H^9 protons of the benzene rings are singlet with 4H intensity (as in **3** ^{1}H NMR spectrum, Table 1 and Figure S1) and they are observed at δ 7.80. The H^{15} and H^{18} protons of the $(HAP)^+$ ring are doublet-doublet with 1H intensities and they are observed at δ 6.50 (H^{15}, $^3J_{H15\text{-}16}$ 8.41 Hz, $^4J_{H15\text{-}17}$ 2.84 Hz) and 7.90 (H^{18}, $^3J_{H18\text{-}17}$ 5.07 Hz, $^4J_{H18\text{-}16}$ 2.38 Hz). The H^{16} and H^{17} protons of the $(HAP)^+$ ring are triplet-doublet with 1H intensities and they are observed at δ 6.50 (H^{16}, $^3J_{H16\text{-}17,15}$ 8.72 Hz, $^4J_{H16\text{-}18}$ 2.69 Hz) and 7.42 ($^3J_{H17\text{-}16,18}$ 7.20 Hz, $^4J_{H17\text{-}15}$ 2.98 Hz). The H^{13} proton of the **6** was not observed in the ^{1}H NMR spectrum. The H^6, H^{12} and H^{19} protons of the **6** are singlets as expected and arisen at δ 11.30 with 1H intensity, δ 7.30 with 2H intensity and δ 6.20 with 2H intensity, respectively. These protons (H^6, H^{12}, H^{13} and H^{19}) were not observed in the ^{1}H NMR spectrum obtained in DMSO-d_6 with D_2O due to deuterium exchange (Figure S4b).

In ^{1}H NMR spectrum of **9** (Figure S6a, Table 3), the H^3 and H^4 protons of the **3** are doublets with 1H intensities and they are observed at δ 6.49 (H^3, $^3J_{H3\text{-}H4}$ 12.00 Hz) and 6.34 (H^4, $^3J_{H4\text{-}H3}$ 11.90 Hz, indicating trans position of H^3 and H^4). The symmetrical H^8 and H^9 protons of the benzene rings are singlet with 4H intensity (as in **3** ^{1}H NMR spectrum, Table 1 and Figure S1) and they are observed at δ 7.80. The symmetrical H^{16} and H^{17} protons of the $(HBI)^+$ ring are doublet-doublet with 2H intensities and they are observed at δ 7.60 (H^{16}, $^3J_{H16\text{-}H17}$ 5.98 Hz, $^4J_{H16\text{-}H17'}$ 2.99 Hz) and 7.20 (H^{17}, $^3J_{H17\text{-}H16}$ 6.02 Hz, $^4J_{H17\text{-}H16'}$ 2.86 Hz). The H^{14} and H^{18} proton of the **9** were not observed in the ^{1}H NMR spectrum (as in BI molecule due to tautomerism). The H^6, H^{12} and H^{13} protons of the **9** are singlets as expected and arisen at δ 10.80 with 1H intensity, 7.20 with 2H intensity and 8.20 with 1H intensity, respectively. These protons (H^6, H^{12}, H^{13} and H^{19}) were not observed in the 1H NMR spectrum obtained in DMSO-d_6 with D_2O due to deuterium exchange (Figure S6b) although the hydrate water molecule (H^{19}) was observed in the thermal studies. For both compounds (**6** and **9**) the acidic hydrogen (H^1) were not observed due to the salt formation, although it was distinguishable in **3** ^{1}H NMR spectrum (Figures S1, S4, S6).

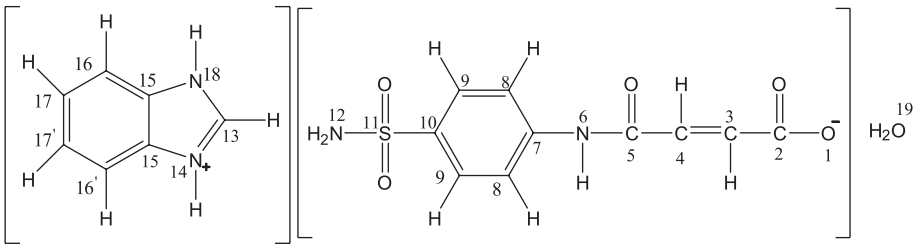
^{13}C NMR spectra of **6** and **9** exhibit thirteen and twelve resonances, respectively (Figures S5 and S7, Tables 2 and 3). Eight peaks out of these resonances δ 168 (C^2), 127 (C^3), 119 (C^4) and 164 (C^5), 139 (C^7), 132 (C^8),

Table 2. ^{1}H NMR and ^{13}C NMR chemical shifts (δ) with coupling constants and assignments for compound **6**



H_3	δ 6.36 (1H, d, $^3J_{H3}$ 12.14 Hz)	C^2	δ 168
H_4	δ 6.33 (1H, d, $^3J_{H4}$ 12.11 Hz)	C^3	δ 127
H_6	δ 11.3 (1H, s)	C^4	δ 119
H_8 H_9	δ 7.80 (4H, s)	C^5	δ 164
H_{12}	δ 7.30 (2H, s)	C^7	δ 139
H_{13}	not observed	C^8	δ 132
H_{15}	δ 6.50 (1H, dxd, $^3J_{H15\text{-}16}$ 8.41 Hz, $^4J_{H15\text{-}17}$ 2.84 Hz)	C^9	δ 132
H_{16}	δ 6.50 (1H, txd, $^3J_{H16\text{-}17,15}$ 8.72 Hz, $^4J_{H16\text{-}18}$ 2.69 Hz)	C^{10}	δ 142
H_{17}	δ 7.42 (1H, txd, $^3J_{H17\text{-}16,18}$ 7.20 Hz, $^4J_{H17\text{-}15}$ 2.98 Hz)	C^{14}	δ 159
H_{18}	δ 7.90 (1H, dxd, $^3J_{H18\text{-}17}$ 5.07 Hz, $^4J_{H18\text{-}16}$ 2.38 Hz)	C^{15}	δ 138
H_{19}	δ 6.20 (2H, s)	C^{16}	δ 109
		C^{17}	δ 112
		C^{18}	δ 147

Table 3. ^{1}H NMR and ^{13}C NMR chemical shifts (δ) with coupling constants and assignments for compound **9**



H_3	δ 6.49 (1H, d, $^{3}J_{H3\text{-}H4}$ 12.00 Hz)	C_2	δ 168
H_4	δ 6.34 (1H, d, $^{3}J_{H4\text{-}H3}$ 11.90 Hz)	C_3	δ 127
H_6	δ 10.80 (1H, s)	C_4	δ 119
H_8, H_9	δ 7.80 (4H, s)	C_5	δ 164
H_{12}	δ 7.20 (2H, s)	C_7	δ 139
H_{13}	δ 8.20 (1H, s)	C_8	δ 131
H_{14}	not observed	C_9	δ 132
H_{16}	δ 7.60 (2H, dxd, $^{3}J_{H16\text{-}H17}$ 5.98 Hz, $^{4}J_{H16\text{-}H17,H17'}$ 2.99 Hz)	C_{10}	δ 142
H_{17}	δ 7.20 (2H, dxd, $^{3}J_{H17\text{-}H16}$ 6.02 Hz, $^{4}J_{H17\text{-}H16,H16'}$ 2.86 Hz)	C_{13}	δ 116
H_{18}	not observed	C_{15}	δ 138
H_{19}	not observed	C_{16}	δ 143
		C_{17}	δ 123

131 (C^{9}) and 142 (C^{10}) could be assigned to the carbons of $SAMAL^{-}$ group of all compunds moiety. Five peaks for **6** at δ 159 (C^{14}), 138 (C^{15}), 109 (C^{16}), 112 (C^{17}) and 147 (C^{18}) could be assigned to the carbons of $(HAP)^{+}$ ring moiety. Four peaks for **9** at δ 116 (C^{13}), 138 (C^{15}), 143 (C^{16}) and 123 (C^{17}) could be assigned to the carbons of $(HBI)^{+}$ ring of moiety.

The room temperature NMR spectra for compounds **6** and **9** indicate clearly the formation of the proton transfer compounds between **3** and **5** or **8** with 1:1 ratio (Figure 1).

FTIR measurements

The infrared spectral data of the starting compounds (**3**, **5** and **8**) and compounds **4**, **6**, **7**, **9**, **10** are given in Table S1. In the high frequency region, weak bands 3013-2854 and 3120-3069 cm^{-1} are attributed to the stretching vibrations of aromatic C–H. There is a broad absorption band at 2900 cm^{-1} attributed to the ν(OH) vibrations of carboxylate group of HSAMAL. This band is not observed in compounds **6** and **9** due to proton transfer to AP or BI and in **4** due to complex formation. There are also broad absorption bands at 3484-3390 cm^{-1} which are attributed to the ν(OH) vibrations of coordinated and uncoordinated water and hydroxide moieties in the compounds **4**, **7**, **9** and **10**. The relatively weak and broad band at 2757-2549 cm^{-1} is attributed to the $\nu(N^{+}–H)$ vibration for **6** from $(HAP)^{+}$ and for **9** from $(HBI)^{+}$ indicating the salt formation. These bands were not observed for the compounds **7** and **10** due to the deprotonation of the salt during the complex formation (Figure 1). NH_2 vibrations of sulfonamides in free HSAMAL (3352 and 3212 cm^{-1}), in **4** (3355, 3306 and 3240 cm^{-1}), in **6** (3432 and 3303 cm^{-1}), in **7** (3351 and 3232 cm^{-1}), in **9** (3365, 3322 and 3266 cm^{-1}) and in **10** (3312 and 3103 cm^{-1}) are observed with similar pattern. The strong C=O vibration at 1695 and 1630 cm^{-1} of HSAMAL are shifted to 1691 and 1654 cm^{-1} for compounds **4**, 1677 and 1633 cm^{-1} for compound **6**, 1692 and 1659 cm^{-1} for compound **7**, 1668 and 1626 cm^{-1} for compound **9** and 1668 and 1602 cm^{-1} for compound **10**. It indicates the role of carboxylic acid on the structures of **4**, **6**, **7**, **9** and carboxylic acid and amid groups on the structure of **10**. The strong absorption bands at the region of 1626-1408 cm^{-1} are attributed to the ν(C=N) and ν(C=C) vibrations for all compounds. In the latter case, $SAMAL^{-}$ was assumed to coordinate to the copper(II) ion from both carbonyl groups. The strong absorption bands for SO_2 groups in HSAMAL in compounds **4, 7** and **10** are observed at the region of 1409-1091 cm^{-1} with similar profiles and almost similar vibrations. The ring wagging vibrations of the pyridine groups are also observed at 768-731 cm^{-1} region for compounds AP and **6** and **7**. The weak bands at 537-555 cm^{-1} and 476-438 cm^{-1} are from the M–N and M–O vibrations of compounds **4**, **7** and **10**.

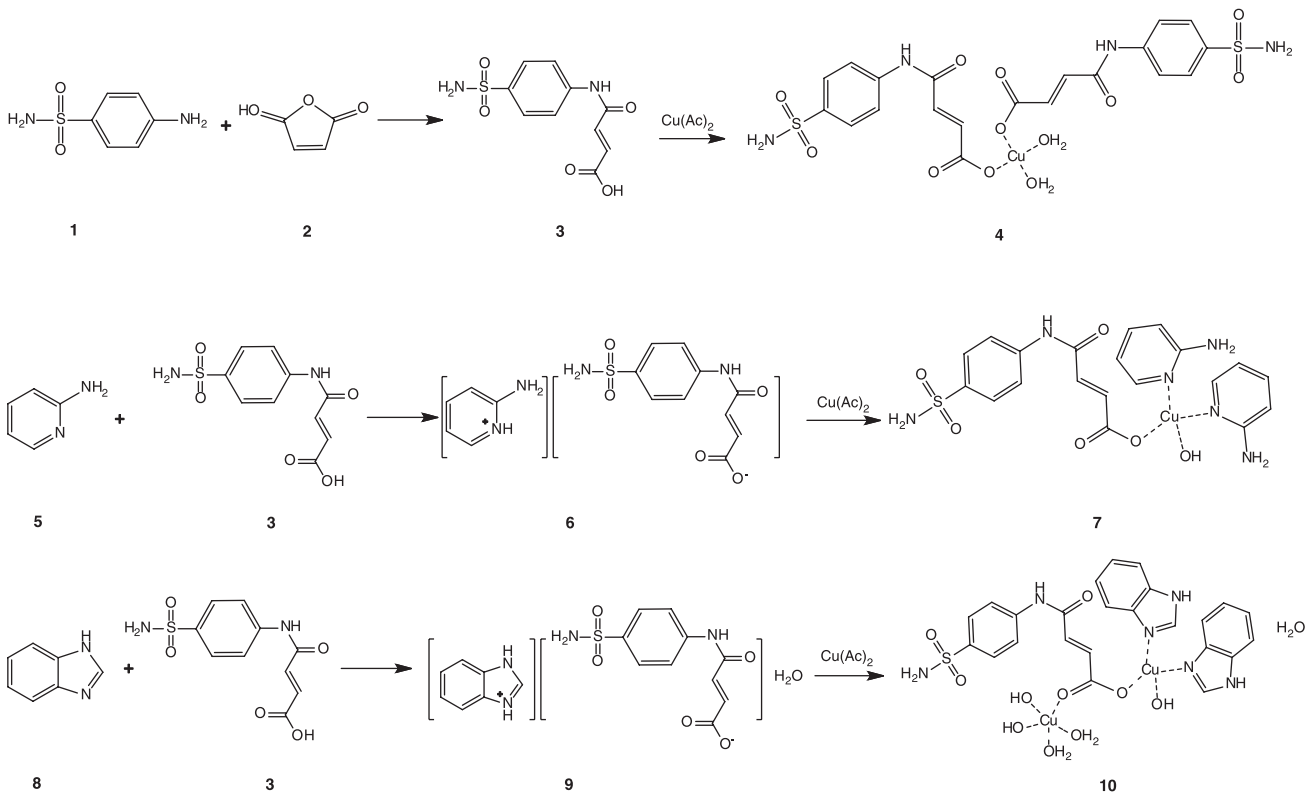


Figure 1. Syntheses of compounds **3**, **4**, **6**, **7**, **9** and **10**.

Thermal analyses of **4**, **6**, **7**, **9** and **10**

Figures S8-S12 show the TG-DTG and DTA curves of compounds **4**, **6**, **7**, **9** and **10** and the thermal analyses results are given in Table S2.

For the compound **6**, two stages are observed and the first endothermic stage corresponds to the loss of $C_5H_9N_3O_2S$ (possibly HAP + SO_2NH_2) unit and the second exothermic one is the decomposition of the residue of $C_{10}H_7NO_3$ unit (possibly $C_6H_4NHCOCHCHCOO$).

For the compound **7**, three stages are observed. The first endothermic peak corresponds to the loss of one mole OH. The second endothermic stage is consistent to the loss of two moles of AP. One mole of SAMAL is decomposed exothermically in the third stage. The final decomposition product is CuO identified by IR spectroscopy.

For the compound **9**, three stages are observed, and the first endothermic stage corresponds to the loss of one mole of water. The second one is endothermic stage, which corresponds to the loss of $C_7H_{10}N_3O_2S$ unit (possibly HBI + SO_2NH_2) and the third second exothermic one is the decomposition of the residue of $C_{10}H_7NO_3$ unit (possibly $C_6H_4NHCOCHCHCOO$).

For the compounds **4** and **10**, three stages are observed, and the first endothermic stage corresponds to the loss of two coordinated water molecules for **4** and one uncoordinated water molecule for **10**. The endothermic second stage is consistent to the loss of 2 moles sulfonamide (SO_2NH_2) for **4** and $C_{12}H_{13}O_5$ units (possibly $2H_2O$ + 3OH + $2C_6H_3$) for **10**. $C_{20}H_{14}N_2O_6$ (possibly 2AP + $C_6H_4NHCOCHCHCOO$) and $C_{12}H_{13}N_6O_5S$ units (possibly SAMAL + $2CH_2N_2$) are also decomposed exothermically in the following stage for **4** and **10**, respectively. The final decomposition products are CuO for **4** and **10** and they are identified by IR spectroscopy.

UV-Vis spectrum, magnetic susceptibility and molar conductivity

The electronic spectra of compounds **4**, **6**, **7**, **9** and **10** and the free ligands **3**, **5** and **8** were recorded in DMSO solution with 1×10^{-3} mol L^{-1} concentrations at room temperature (Table S3). Characteristic π-π* transitions in the range of 283-367 nm (2500-48170 L mol^{-1} cm^{-1}) for **4**, **6**, **7**, **9** and **10** are observed in DMSO. The same π-π* transition profiles are also detected for the free ligands HSAMAL, AP and BI and there is no marked difference from those of either proton transfer compounds or metal complexes. The bands for the d-d transitions in DMSO are observed at 765 nm (150 L mol^{-1} cm^{-1}) for **4**, 761 nm (200 L mol^{-1} cm^{-1}) for **7**, 769 nm (240 L mol^{-1} cm^{-1}) for **10**.

The room temperature magnetic moments of the metal complexes are 1.65 BM for **4**, 1.67 BM for **7** and 1.69 BM

for **10** *per* metal ion, indicating the presence of one (d^9) unpaired electron and absence of metal-metal bond for **10**.

The molar conductivity data in DMSO are 3.8 Ω^{-1} cm^2 mol^{-1} for **4**, 3.0 Ω^{-1} cm^2 mol^{-1} for **7** and 1.8 Ω^{-1} cm^2 mol^{-1} for **10** indicating that the complexes **4**, **7** and **10** are non-ionic[37] and OH^- groups are coordinated to the copper(II) ions.

The very powerful tool single X-ray diffraction studies cannot be applied to identify the structures of complexes **4**, **7** and **10** due to their powdery forms. Using elemental and thermal analyses, the formulas of the complexes were proposed with the aid of spectral (FTIR, UV-Vis) and of magnetic susceptibility and molar conductivity studies (Figure 1).

CA purification and inhibition assay

For achievement of the purpose of the study, hCA I and hCA II isozymes were purified from human erythrocytes and the inhibition potentials of proton transfer salt, simple metal complexes and mixed ligand complexes have been tested on these isozymes. Specific activity was calculated for purified hCA I and II (918.4 EU mg^{-1} for hCA I and 2053.2 EU mg^{-1} for hCA II). hCA I and hCA II isozymes were purified from erythrocytes as 84.40 and 188.71 fold, respectively (Table S4).

Newly synthesized proton transfer salt and mixed ligand complexes, as well as the reference compound AAZ were tested under *in vitro* conditions for their inhibition potentials against hCA I and hCA II (Table 4).

Simple ligands, **5** and **8**, and simple metal complexes, **12** and **13**, did not inhibit the hydratase and esterase activities of hCA I and hCA II isozymes.

The slow cytosolic isoform, hCA I, was moderately inhibited by synthesized compounds for hydratase activity. Compounds **4**, **6**, **7**, **9** and **10** have weaker inhibition potentials than AAZ. Among the synthesized compounds, the complex **4** has most powerful inhibition effect on the hydratase activity of hCA I (IC_{50} values 13.34 µM, Table 4). Unlike the hydratase activity, **3** and compounds **4**, **6**, **7**, **9** and **10** have comparable inhibition potential with AAZ for esterase activity. Generally, proton transfer salts and mixed ligand complexes have stronger inhibition effects than simple ligands and simple metal complexes on the esterase activity of hCA I. Regarding to K_i values, it is shown that the proton transfer salt (**6**) and its metal complex (**7**) are stronger inhibitor than the other proton transfer salt (**9**) and its metal complex (**10**) (Table 4). For esterase activity of hCA I, **6** has shown the highest inhibition effect (esterase IC_{50} value 0.57 ± 0.002 µmol L^{-1} and K_i value 0.18 ± 0.007 µmol L^{-1}). Inhibition potentials were in the order of AAZ > **6** > **7** ≈ **9** > **10** > **4** > **3** according to esterase IC_{50} values, and **6** > AAZ > **7** > **9** > **10** ≈ **4** > **3** according to K_i values.

Similar to hCA I, the faster and physiologically dominant isoform hCA II was moderately inhibited by synthesized compounds for hydratase activity. However inhibition effects of synthesized compounds are higher for the hydratase activity of hCA II than the hydratase activity of hCA I. As seen from Table 4, the inhibition potentials of compounds

Table 4. The inhibition data and K_i values of hCA I and hCA II isozymes for hydratase and esterase activity

Compound	Hydratase IC_{50}[b,c] / (µmol L^{-1})		Esterase IC_{50}[b,c] / (µmol L^{-1})		K_i[b,c] / (µmol L^{-1})	
	hCA I	hCA II	hCA I	hCA II	hCA I	hCA II
AAZ[a]	0.39 ± 0.008	0.20 ± 0.005	0.42 ± 0.004	0.31 ± 0.008	0.26 ± 0.003	0.14 ± 0.005
1	30.44 ± 0.008	5.67 ± 0.003	28.14 ± 0.012	5.36 ± 0.005	26.32 ± 0.009	4.14 ± 0.011
2	no inhibition	no inhibition	no inhibition	no inhibition	no inhibition	no inhibition
3	84.07 ± 0.012	56.48 ± 0.009	1.64 ± 0.005	1.51 ± 0.009	1.02 ± 0.004	0.93 ± 0.006
4	13.34 ± 0.005	10.58 ± 0.008	1.43 ± 0.007	1.03 ± 0.006	0.98 ± 0.013	0.91 ± 0.003
5	no inhibition	no inhibition	no inhibition	no inhibition	no inhibition	no inhibition
6	49.57 ± 0.008	28.40 ± 0.007	0.57 ± 0.002	0.42 ± 0.011	0.18 ± 0.007	0.13 ± 0.008
7	21.76 ± 0.009	18.44 ± 0.010	0.80 ± 0.003	0.25 ± 0.003	0.30 ± 0.005	0.12 ± 0.004
8	no inhibition	no inhibition	no inhibition	no inhibition	no inhibition	no inhibition
9	50.87 ± 0.010	22.07 ± 0.013	0.81 ± 0.003	0.72 ± 0.002	0.56 ± 0.004	0.35 ± 0.002
10	24.11 ± 0.003	23.01 ± 0.006	1.36 ± 0.005	1.19 ± 0.008	0.97 ± 0.002	0.86 ± 0.001
12	no inhibition	no inhibition	no inhibition	no inhibition	no inhibition	no inhibition
13	no inhibition	no inhibition	no inhibition	no inhibition	no inhibition	no inhibition
$Cu(Ac)_2.H_2O$	14.21 ± 0.004	145.71 ± 0.028	11.25 ± 0.010	141.13 ± 0.024	10.24 ± 0.014	130.11 ± 0.021

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

4, **6**, **7**, **9** and **10** are higher at least two-fold than simple ligand, **3**. Similar to hCA I, **4** is the most potent inhibitor for the hydratase activity of hCA II (10.58 ± 0.008 μM, Table 4). Newly synthesized compounds exhibited similar effects to hCA I on esterase activity of this isoform. Compound **7** (esterase IC_{50} = 0.25 ± 0.003 μmol L^{-1} and K_i 0.12 ± 0.004 μmol L^{-1} for hCA II) have shown effective inhibition on the isozyme. When compared the inhibition effects of synthesized compounds, it is shown that **6** and **7** is more effective than others. AP and BI moieties in proton transfer salts and mixed ligand complexes have changed inhibition effects of compounds. But it is rather difficult to rationalize these data as no X-ray crystal structures of CA-inhibitor interactions. Inhibition potentials were in the order of **7** > AAZ > **6** > **9** > **4** > **10** > **3** according to esterase IC_{50} values, and **7** ≈ **6** ≈ AAZ > **9** > **10** > **4** > **3** according to K_i values.

In summary, synthesized compounds have pharmacologically remarkable inhibition potentials on hCA I and hCA II. These compounds can be the candidate agents for further studies of glaucoma therapy.

Conclusions

In this present work, two novel proton transfer salts **(6** and **9)** of maleic acid sulfonamide, three copper(II) complexes **(4, 7** and **10)** were prepared for the first time. The formulas of **4**, **7** and **10** have been proposed by the results of elemental, spectral, magnetic measurement, molar conductivity and thermal analyses.

These newly synthesized proton transfer salts and their metal complexes possess more remarkable inhibition effects on hCA I and on hCA II for esterase activities than those of found for hydratase activities, and thus might be considered as possible candidate agents for *in vivo* therapeutic studies of glaucoma.

Supplementary Information

Supplementary data are available free of charge at http://jbcs.sbq.org.br as PDF file.

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