

CARBONIC ANHYDRASE INHIBITORY ACTIVITIES OF NOVEL PROTON TRANSFER SALTS AND THEIR Cu(II) COMPLEXES

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In this study, two new proton transfer salts of sulfonamide derivatives of maleic acid, namely (ClHabt)⁺(mabsmal)⁻ (**1**) and (ClHabt)⁺(pabsmal)⁻ (**2**), were obtained from 2-amino-6-chlorobenzothiazole (Clabt) and *N*-(3-sulfamoylphenyl)maleamide acid (Hmabsmal) and *N*-(4-sulfamoylphenyl)maleamide acid (Hpabsmal), respectively. Also, the Cu(II) complexes (**3** and **4**) of salts (**1** and **2**) and of Hmabsmal (**5**) were prepared. Compounds **1–5** were characterized by elemental, NMR (¹H and ¹³C), FTIR, and thermal analyses, as well as UV-Vis, magnetic moment, and molar conductivity measurements. Carbonic anhydrase isoenzymes (hCA I and II) were purified from human erythrocyte cells by affinity chromatography. The effects of the synthesized compounds on the hydratase and esterase activities of CA isoenzymes were studied *in vitro*. The results reveal that the synthesized compounds inhibit both esterase and hydratase activities of hCA I and hCA II. The inhibition constants of the compounds (*K_i*) were determined according to the esterase activity measurements. *K_i* values of **1–5** are in the range of 0.06 ± 0.003 μM and 4.25 ± 0.100 μM for hCA I, and of 0.02 ± 0.001 μM and 3.21 ± 0.200 μM for hCA II.

Keywords: 2-amino-6-chlorobenzothiazole; *N*-(3/4-sulfamoylphenyl)maleamide acids; proton transfer salt; Cu(II) complexes; carbonic anhydrase inhibition

ИНХИБИТОРНА АКТИВНОСТ ВРЗ КАРБОНСКАТА АНХИДРАЗА НА НОВИ СОЛИ СО ТРАНСФЕР НА ПРОТОНИ И НИВНИТЕ Cu(II)-КОМПЛЕКСИ

Во оваа студија, од 2-амино-6-хлорбензотиазол (Clabt) и соодветно на *N*-(3-сулфамоилфенил)малеамидна киселина (Hmabsmal) и *N*-(4-сулфамоилфенил)малеамидна киселина (Hpabsmal), се добиени две нови протон трансфер соли на сулфонамидни деривати на малеинска киселина, имено (ClHabt)⁺(mabsmal)⁻ (**1**) и (ClHabt)⁺(pabsmal)⁻ (**2**). Исто така беа приготвени Cu(II) комплекси (**3** и **4**) на солите (**1** и **2**) и на Hmabsmal (**5**). Соединенијата **1–5** беа карактеризирани со елементарна, NMR (¹H и ¹³C), FTIR и термичка анализа, како и со UV-Vis и мерења на магнетниот момент и на моларната спроводливост. Изоензимите на карбонска анхидраза (hCA I и II) беа пречистени од човечки еритроцитни клетки со афинитетна хроматографија. Ефектите на синтетизираните соединенија на активноста на хидратазата и на естераза на СА-изоензимите беа проучени *in vitro*. Резултатите откриваат дека синтетизираните соединенија ја инхибираат активноста и на естеразата и на хидратазата на hCA I и на hCA II. Инхибиторните константи на соединенијата (*K_i*) беа определени со мерења на активноста на естеразата. Вредностите на *K_i* на **1–5** се во опсегот од 0,06 ± 0,003 μM и 4,25 ± 0,100 μM за hCA I и од 0,02 ± 0,001 μM и 3,21 ± 0,200 μM за hCA II.

Клучни зборови: 2-амино-6-хлорбензотиазол; *N*-(3/4-сулфамоилфенил)малеамидни киселини; соли со трансфер на протони; Cu(II) комплекси; инхибиција на карбонска анхидраза

1. INTRODUCTION

Sulfonamides and 2-aminobenzothiazoles perform a wide range of various biological activities, such as anti-HIV protease¹ as well as anti-inflammatory,² antiglaucoma,³ antibacterial, anti-fungal,⁴ antiviral, and antitumor actions.⁵ Carbonic anhydrases (CAs, EC 4.2.1.1) are present in almost all living organisms as metalloenzymes catalyzing the hydration of carbon dioxide to bicarbonate and a proton.^{6,7} CAs are encoded by eight different independent gene families (α -, β -, γ -, δ -, ζ -, η -, θ -, and ι -).⁸ α -CAs contain Zn^{2+} in their active site; they are contained in mammals and vertebrates, whereas sixteen different isozymes of them are present in humans (hCAs).⁹ The hCA I is a major cytosolic carbonic anhydrase present in erythrocytes' gastrointestinal tract and eyes.¹⁰ It is physiologically relevant to retinal edema and cerebral edema.¹¹ The hCA II is the most active isoform among the α -CAs, present in erythrocytes' eye, gastrointestinal tract, bone osteoclasts, kidney, lung, testes, and brain, and it is physiologically relevant to glaucoma, edema, epilepsy, and altitude sickness.¹² Just these two isoforms are associated with various physiological disorders. This shows how important these enzymes are from a pharmacological point of view. Primary sulfonamides are the most well-known and clinically used inhibitors of CA isoenzymes. However, since the isoform selectivity of these compounds is weak, their side effects are considerably higher. In order to overcome the isoform selectivity problem, researchers are currently trying to develop inhibitors that would bind to different moieties in the active site or derivatize the primary sulfonamides that bind to Zn^{2+} in such a way that they ought to interact with different amino acids in the active site. A primary sulfonamide derivative coded SLC-0111,¹³ which has been in clinical trials in recent years, is an effective example of an isoform-selective sulfonamide derivative strategy.

Also, the Cu(II) complexes of carboxylic acids with 2-aminobenzothiazole have been extensively studied in recent years.¹⁴⁻¹⁶ The metal complexes of proton transfer salts have shown better biological activities than free ligands and salt.^{2,3} The simple transition metal complexes of *Hmabsmal* have not been synthesized; nevertheless, there are a few reports on the synthesis of proton transfer salts and mixed ligand metal complexes {Co(II) and Cu(II)} of *Hmabsmal* with other ligands, such as 2-aminopyridine derivatives.^{17,18} Fe(II), Co(II), and Cu(II) complexes of *Hpabsmal*, proton transfer salts of *Hpabsmal* with 2-aminopyridine or 1*H*-

benzimidazole, and the Co(II), Ni(II), Cu(II) and Zn(II) complexes have been synthesized earlier and reported in our laboratories.¹⁷⁻¹⁹

In this study, relying on the importance of the sulfonamide compounds in the inhibition of CA, two new proton transfer salts (ClHabt)⁺(*mabsmal*)⁻ (**1**) and (ClHabt)⁺(*pabsmal*)⁻ (**2**) were obtained from the reaction between Clabt (the base) and *Hmabsmal* or *Hpabsmal* (the acid), respectively. The Cu(II) complexes (**3** and **4**) of salts (**1** and **2**) and of *Hmabsmal* (**5**) were characterized by spectroscopic methods. The potential use of these compounds as new inhibitors of hCA I and hCA II isoenzymes was also investigated.

2. EXPERIMENTAL SECTION

2.1. Synthesis of 1-5

The materials and equipment used in our experimental study are provided in a supplementary file. The starting compounds are the acids, *Hmabsmal*²⁰ and *Hpabsmal*,²¹ which were synthesized according to the literature. The spectroscopic data of *Hmabsmal* and *Hpabsmal* are given in Tables S1 and S2 for ¹H and ¹³C NMR, in Table S3 for FT-IR, and in Table S5 for UV-Vis, whereas Figures S1-S4 render the NMR spectra.

A solution of 0.920 g Clabt in 35 ml absolute ethanol was added to the solution of 1.35 g maleic acid (*Hmabsmal* for **1** or *Hpabsmal* for **2**) in 35 ml of absolute ethanol. The mixture was stirred at room temperature for two days in order to obtain white solids of **1** and **2** (1.934 g, 85 % yield for **1**; 1.817 g, 80 % yield for **2**).

1 mmol of copper(II) acetate monohydrate and 1 mmol proton transfer salt {**1** (0.420 g) for **3** and **2** (0.455 g) for **4**} and 1 mmol *Hmabsmal* (0.270 g) were dissolved in technical ethanol (50%) (50 ml) with stirring for two days. The mixture was induced to give a solid powder 0.358 g (blue, 65 % yield) for **3**, 0.258 g for **4** (green, 70 % yield), and 0.246 g for **5** (blue, 75% yield) (Fig. 1).

Anal. Calcd. for **1** (C₁₇H₁₅N₄O₅S₂): C, 44.88%; H, 3.32%; N, 12.32%; S, 14.10%. Found: C, 44.90%; H, 3.55%; N, 12.30%; S, 14.05%; for **2** (C₁₇H₁₅N₄O₅S₂): C, 44.88%; H, 3.32%; N, 12.32%; S, 14.10%. Found: C, 44.85%; H, 3.52%; N, 12.35%; S, 14.08%; for **3** (C₃₄H₃₄N₈O₁₃S₄Cl₂Cu): C, 39.83%; H, 3.34%; N, 10.93%; S, 12.51%; Cu, 6.20%. Found: C, 39.85%; H, 3.35%; N, 10.90%; S, 12.50%; Cu, 6.50%; for **4** (C₂₄H₂₂N₆O₇S₃Cl₂Cu): C, 39.11%; H, 3.01%; N, 11.40%; S, 13.05%; Cu, 8.62%. Found: C, 39.10%; H, 3.05%; N, 11.38%; S, 13.10%; Cu, 8.63%; for **5** (C₂₀H₂₄N₄O₁₃S₂Cu):

C, 36.61%; H, 3.69%; N, 8.54%; S, 9.77%; Cu, 8.54%. Found: C, 39.65%; H, 3.65%; N, 8.55%; S, 9.80%; Cu, 8.60%.

In addition, the Cu(II) metal complexes of H_pabsmal (CuH_pabsmal)¹⁸ and Clabt (CuClabt)²² were prepared according to the literature.

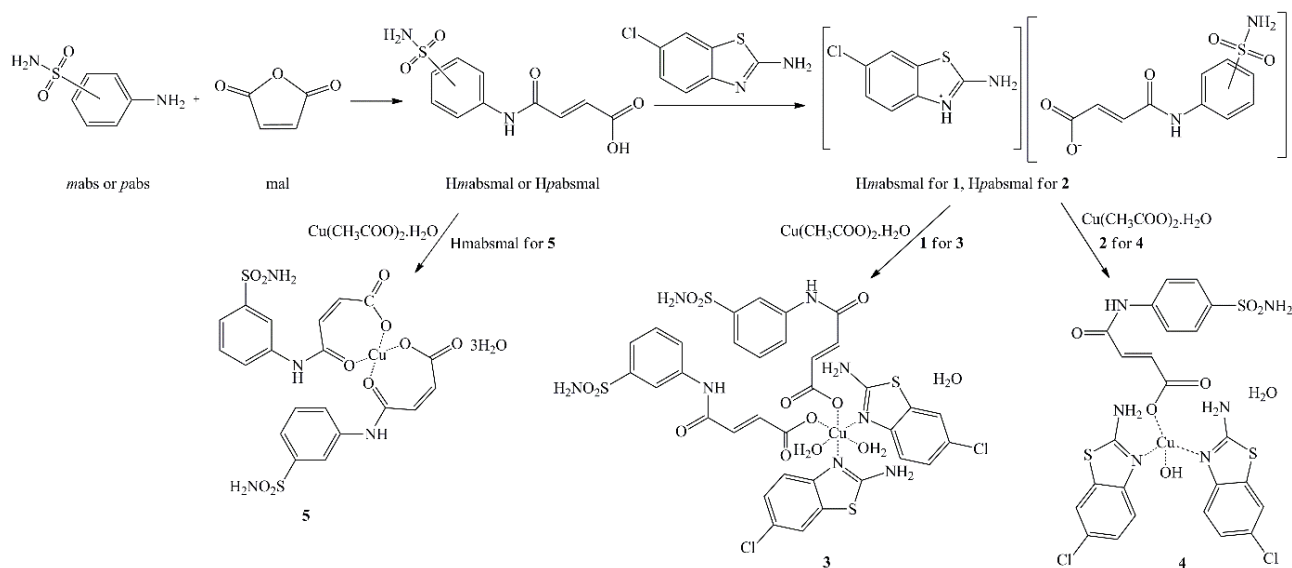


Fig. 1. Syntheses of compounds 1–5

2.2. Purification of hCA I and hCA II

The purification of the enzymes was described in detail in our previous studies.^{15–19} To review, blood samples collected in anticoagulated tubes were centrifuged, while erythrocytes were separated and hemolyzed. Following centrifugation, the supernatant, whose pH had been adjusted to 8.7, was loaded onto a Sepharose®-4B-L-tyrosine-*p*-aminobenzenesulfonamide column. After extensive washing, the hCA I and II isoenzymes were eluted with 1.0 M NaCl/25.0 mM Na₂HPO₄ (pH 6.3) and 0.1 M CH₃COONa/0.5 M NaClO₄ (pH 5.6).²³ Protein quantity was determined in the eluates.²⁴ The purified enzymes were characterized by SDS-PAGE analysis in the presence of standard hCA I and hCA II.²⁵ (Table S6, Fig. S5).

2.3. Hydratase and esterase activity assay and the determination of the IC₅₀ values and inhibition constants (K_i)

Hydratase and esterase activities of purified hCA I and hCA II were determined according to the literature.^{26,27} The activity measurement was described in detail in our previous studies.^{15–19}

The IC₅₀ values were determined by measuring hydratase and esterase activity in the presence of the following compounds: free ligands, simple Cu(II) complexes, and 1–5. The regression analysis graphs were drawn by plotting inhibitor concentrations against percent enzyme activity using the Microsoft Excel Package Program (Microsoft Office 2016).

The method used to determine the K_i values was described in detail in our previous studies.^{15–19} In short, in order to determine the K_i values, esterase activity measurements were made at five different substrate concentrations for each of the three different inhibitor concentrations (30 %, 50 %, and 70 % inhibition effect). The data were linearized with a Lineweaver-Burk plot in order to obtain K_i values.²⁸

The IC₅₀ and K_i values were determined by averaging the results from triplicate experiments.

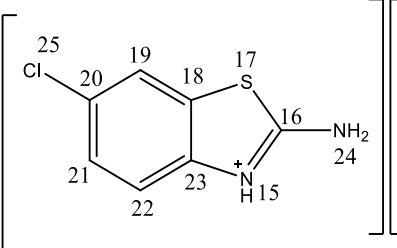
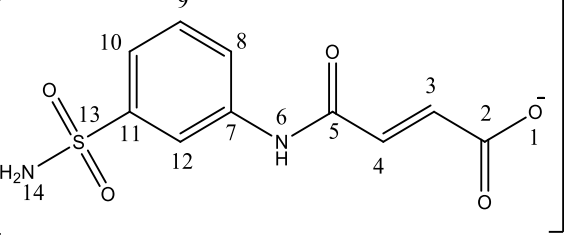
3. RESULTS AND DISCUSSION

3.1. NMR studies of 1 and 2

The NMR spectra (¹H and ¹³C) of 1 and 2 are given in d₆-DMSO with and without D₂O in Figures S6–S9, whereas the chemical shift values can be seen in Tables 1 and 2.

Table 1

 ^1H NMR and ^{13}C NMR results for compound **1**

			
H ³	6.50 (1H, d) [³ J _{H3-H4} = 11.82 Hz]	C ²	167.37
H ⁴	6.35 (1H, d) [³ J _{H4-H3} = 11.78 Hz]	C ³	131.39
H ⁶	11.60 (1H, s)	C ⁴	131.93
H ⁸	7.55 (1H, d) [³ J _{H8-H9} = 7.34 Hz]	C ⁵	164.00
H ⁹	7.65 (1H, t) [³ J _{H9-H8,10} = 7.17 Hz]	C ⁷	139.52
H ¹⁰	7.35 (1H, d) [³ J _{H10-H9} = 7.54 Hz]	C ⁸	121.32
H ¹²	8.35 (1H, s)	C ⁹	130.84
H ¹⁹	7.75 (1H, s)	C ¹⁰	129.99
H ¹⁴ , H ²¹ , H ²² , H ²⁴	7.40-7.60 (6H, m)	C ¹¹	145.20
		C ¹²	116.88
		C ¹⁶	166.89
		C ¹⁸	130.84
		C ¹⁹	121.27
		C ²⁰	122.67
		C ²¹	125.88
		C ²²	118.19
		C ²³	153.25

In the ^1H NMR spectrum of **1** at room temperature (Fig. S6a, Table 1), the H³ and H⁴ protons of **1** are observed at 6.50 ppm (H³, doublet, $^3J_{\text{H}3-\text{H}4}$ = 11.82 Hz) and 6.33 ppm (H⁴, doublet, $^3J_{\text{H}4-\text{H}3}$ = 11.78 Hz) with 1H intensities, indicating the transposition of H³ and H⁴. The H⁸, H⁹, H¹⁰ and H¹² protons of **1** are detected at 7.55 ppm (H⁸, doublet, $^3J_{\text{H}8-\text{H}9}$ = 7.34 Hz), 7.35 ppm (H¹⁰, triplet, $^3J_{\text{H}10-\text{H}9}$ = 7.54 Hz), 7.65 ppm (H⁹, doublet, $^3J_{\text{H}9-\text{H}8,10}$ = 7.17 Hz), and 8.35 ppm (H¹², singlet) with 1H intensities, respectively. H¹⁴, H²¹, H²², and H²⁴ protons of the **1** are observed in the range of 7.40–7.60 ppm (multiplet) with 6H intensities. The H⁶ and H¹⁹ protons of the **1** are observed as singlets at 11.60 ppm with 1H intensity and 7.75 ppm with 1H intensity, respectively.

In the ^1H NMR spectrum of **2** at room temperature (Fig. S8a, Table 2), the H³ and H⁴ protons of **2** are detected at 6.35 ppm (H³, doublet, $^3J_{\text{H}3-\text{H}4}$ = 11.74 Hz) and 6.50 ppm (H⁴, doublet, $^3J_{\text{H}4-\text{H}3}$ = 11.74 Hz) with 1H intensities, indicating the transposition of H³ and H⁴. The H⁸, H⁹, H¹¹, H¹², and H¹⁹ protons of the **2** are observed at 7.80 ppm (singlet) with 5H intensities. The H²¹ and H²² protons of **2** are observed at 7.23 ppm (H²¹, doublet, $^3J_{\text{H}21-\text{H}22}$ = 7.74 Hz) and 7.32 ppm (H²², doublet, $^3J_{\text{H}22-\text{H}21}$ = 8.20 Hz) with 1H intensity. The H⁶, H¹⁴, and H²⁴ protons of **2** are observed as singlets at 11.60 ppm with 1H intensity, 7.60 ppm with 2H intensity, and 7.27 ppm with 2H intensity, respectively.

The H¹⁵ protons of **1** and **2** were not detected in the ^1H NMR spectrum. This can be due to the mobility of the proton between the position O1 and N15. H⁶, H¹⁴ and H²⁴ protons of **1** and **2** were not detected in the spectra obtained in d₆-DMSO after D₂O addition due to the deuterium exchange (Figs. S6b and S8b). For both compounds (**1** and **2**), the –COOH hydrogen (H¹) was not observed due to the salt formation and proton transfer, although it was distinguishable in the *H*_mabsmal and *H*_pabsmal ^1H NMR spectra (12.95 and 12.90 ppm) (Figs. S1 and S3).

The ^{13}C NMR spectra of **1** and **2** exhibit seventeen and fifteen resonances (Tables 1 and 2, Figs. S7 and S9) as expected. Seven peaks out of these resonances for **1** and **2**, particularly 166.89 and 167.37 ppm (C¹⁶), 130.84 and 130.81 ppm (C¹⁸), 121.27 and 121.01 ppm (C¹⁹), 122.67 and 124.98 ppm (C²⁰), 125.88 and 126.02 ppm (C²¹), 118.19 and 119.05 (C²²), and 153.25 and 152.21 ppm (C²³), can be designated to the carbons of

HClabt⁺ groups. The other ten peaks for **1** at 167.37 ppm (C²), 131.39 ppm (C³), 131.93 ppm (C⁴), 164.00 ppm (C⁵), 139.52 ppm (C⁷), 121.32 ppm (C⁸), 130.84 ppm (C⁹), 129.99 ppm (C¹⁰), 145.20 ppm (C¹¹) and 116.88 ppm (C¹²) can be designated to the carbons of *mab*smal⁻ ring moiety. The rest of the peaks for **2** at 167.61 ppm (C²),

131.98 ppm (C³), 133.06 ppm (C⁴), 164.13 ppm (C⁵), 139.25 ppm (C⁷), 127.20 ppm (C⁸, C¹²), 119.46 ppm (C⁹, C¹¹), and 142.08 ppm (C¹⁰) can be designated to the carbons of *pab*smal⁻ ring moiety.

The NMR spectra clearly indicate the formation of the salts between Clabt and *Hmab*smal or *Hpab*smal with 1:1 ratio for **1** and **2** (Fig. 1).

Table 2

¹H NMR and ¹³C NMR results for compound **2**

H ³	6.35 (1H, d) [³ J _{H3-H4} = 11.74 Hz]	C ²	168
H ⁴	6.50 (1H, d) [³ J _{H4-H3} = 11.74 Hz]	C ³	139
H ⁶	11.60 (1H, s)	C ⁴	130
H ⁸ , H ⁹ , H ¹¹ , H ¹² , H ¹⁹	7.80 (5H, s)	C ⁵	164
H ¹⁴	7.60 (2H, s)	C ⁷	132
H ²¹	7.23 (1H, d) [³ J _{H21-H22} = 7.74 Hz]	C ⁸ , C ¹²	127
H ²²	7.32 (1H, d) [³ J _{H22-H21} = 8.20 Hz]	C ⁹ , C ¹¹	119
H ²⁴	7.27 (2H, s)	C ¹⁰	142
		C ¹⁶	167
		C ¹⁸	133
		C ¹⁹	119
		C ²⁰	124
		C ²¹	126
		C ²²	121
		C ²³	152

3.2. FT-IR results

The infrared spectral data of the free ligands (*Hmab*smal, *Hpab*smal and Clabt) and **1–5** are given in Table S3. The ν(OH) vibrations of *Hmab*smal and *Hpab*smal were observed as a broad band at 2900 cm⁻¹, but these bands are not observed in **1** and **2** due to the proton transfer to Clabt and in **3** and **4** due to complex formation. The ν(OH) vibration bands, uncoordinated for **3** and **5** and coordinated water for **3–4** and hydroxide moieties **4**, are in the range of 3544–3453 cm⁻¹. The band of the ν(N⁺–H) vibration indicating the salt formation for **1** and for **2** (HClabt⁺) is observed in the range of 2759–2462 cm⁻¹. These bands were not observed for **3** and **4** due to the deprotonation of the salt after the complex formation (Fig. 1). The N–H vibrations of –SO₂NH₂, –CONH–, and –NH₂ in Clabt (3453 and 3265), in *Hmab*smal (3315, 3229 and

3205 cm⁻¹), in *Hpab*smal (3352, 3263, and 3212 cm⁻¹), in **1** (3392, 3339, 3313, 3282, and 3187 cm⁻¹), in **2** (3388, 3304, 3261, 3239, and 3207 cm⁻¹), in **3** (3385, 3223, 3304, 3271, and 3244 cm⁻¹), in **4** (3391, 3256, 3240, and 3174 cm⁻¹) and in **5** (3331, 3261, 3294, 3234, and 3211 cm⁻¹) are survey with similar texture. The strong C=O vibrations at 1688 and 1620 cm⁻¹ of *Hmab*smal and at 1695 and 1630 cm⁻¹ of *Hpab*smal change for the salts to 1665 and 1620 cm⁻¹ for **1**, and 1658 and 1612 cm⁻¹ for **2**, whereas for the complexes, to 1641 and 1617 cm⁻¹ for compound **3**, 1691 and 1641 cm⁻¹ for **4**, and 1666 and 1600 cm⁻¹ for **5**. This indicates the formation of salts and complexes. The strong absorption bands of all compounds were detected in the range of 1401–1627 cm⁻¹ for ν(C=N) and ν(C=C), 1264–1142 cm⁻¹ for SO₂ groups, except Clabt, and 1397–1054 cm⁻¹ for C–O, except Clabt. The weak bands at 3095–3040 cm⁻¹ are from the aromatic C–H

and conjugated H–C=C–H vibrations of all compounds, 550 cm^{-1} and 585 cm^{-1} are from the M–N vibrations of **3** and **4**, and 454 cm^{-1} , 498 cm^{-1} , and 462 cm^{-1} are from the M–O vibrations of **3–5**, respectively.

3.3. Thermal analyses result of **3–5**

The TG, DTA, and DTG curves of the compounds **3–5** are shown in Figures S10–S12 and the results of the thermal analyses are given in Table S4.

For **3–5**, in the first stage, the endothermic peaks correspond to the loss of three moles water for **3**, one mole water and one mole OH for **4**, and three moles water for **5**. In the second stage, the endothermic peaks for **3** and **5** and the exothermic peak for **4** are consistent with the loss of $\text{C}_{18}\text{H}_{18}\text{N}_4\text{O}_6\text{S}_2$ for **3**, $\text{C}_{10}\text{H}_{12}\text{N}_2\text{O}_4\text{S}_2$ for **4**, and $\text{C}_5\text{H}_6\text{NO}_2\text{S}$ unit for **5**. In the third stage, the exothermic peaks are the decomposition of the residue of $\text{C}_{16}\text{H}_{10}\text{N}_4\text{O}_4\text{S}_2\text{Cl}_2$ for **3**, $\text{C}_4\text{H}_2\text{N}_2\text{O}_3$ for **4**, and a $\text{C}_{19}\text{H}_{14}\text{N}_5\text{O}_3\text{S}_2\text{Cl}_2$ unit for **5**. The final decomposition product is CuO for **3–5**.

3.4. UV spectra results

The UV spectra values of all compounds are shown in DMSO solution at $1 \cdot 10^{-3} \text{ mol l}^{-1}$ concentrations (Table S5). π - π^* transitions in the range of 297–288 nm (34550 – $19990 \text{ l mol}^{-1} \text{ cm}^{-1}$) are observed for **1–5**. The π - π^* transitions are observed for the free ligands Clabt, Hmabsmal, and Hpabsmal, and there are no significant differences from those of **1–5**. The bands for d-d transitions of compounds (**3–5**) are observed at 767, 782, and 763 nm (13 , 40 , and $36 \text{ l mol}^{-1} \text{ cm}^{-1}$), respectively.

3.5. Magnetic susceptibility

The results of the magnetic moments of compounds **3–5** are 1.64, 1.63, and 1.65 BM, respectively, which reveals the presence of one unpaired (Cu(II), d^9) electron.

3.6. Molar conductivity

The molar conductivity data of the compounds **3–5** in DMSO are 2.5, 6.7, and $11.2 \Omega^{-1}$

$\text{cm}^2 \text{ mol}^{-1}$, which indicates that all complexes are non-ionic.²⁹

3.7. CA inhibition assay

In this part of the study, the effects of all compounds on human cytosolic carbonic anhydrase isoenzymes, hCA I and hCA II, were examined *in vitro*. The inhibitory effects on carbonic anhydrase isoenzymes were observed as expected due to the sulfonamide group present in the compounds. Both hydratase and esterase activities of hCA I and hCA II are inhibited by the synthesized compounds (Fig. 2).

The inhibition of the hydratase activity shows that the compounds interact (directly or indirectly) with the zinc ion in the active site of the isoenzymes.³⁰ Sulfonamide derivative Hpabsmal is a less potent inhibitor than pabs, but the Cu(II) complex of Hpabsmal proves to be a more effective inhibitor than pabs and Hpabsmal for the hydratase activities of the isoenzymes. The proton transfer salt of the Hpabsmal compound (**2**, $\text{HClabt}^+\text{pabsmal}^-$) is a more effective inhibitor than pabsmal, Hpabsmal, and CuHpabsmal compounds. It is estimated that the inhibition effect of compound **2** emerges from the presence of the HClabt^+ ion in its structure. The Cu(II) complex of **2** (**4**) shows nearly four times better inhibition effects on the hydratase activities of the isoenzymes as compared to **2**. The fact that mabs and its derivatives, Hmabsmal and **1**, did not have any inhibitory effects on hydratase activity indicates that the enzyme-inhibitor interaction was significantly weakened in the presence of $-\text{SO}_2\text{NH}_2$ at the *meta* position. The inhibitory property of compounds **3** and **5** shows that these compounds, which are bulkier than the starting compounds (Hmabsmal and **1**), interact more robustly with the active site. Among the synthesized compounds, the most potent hydratase inhibitor is **3** (Table 3). This compound, which is the most voluminous compound in our study, can easily interact with both the hydrophilic and hydrophobic parts of the active site. This is due to its hydrophilic and hydrophobic groups.

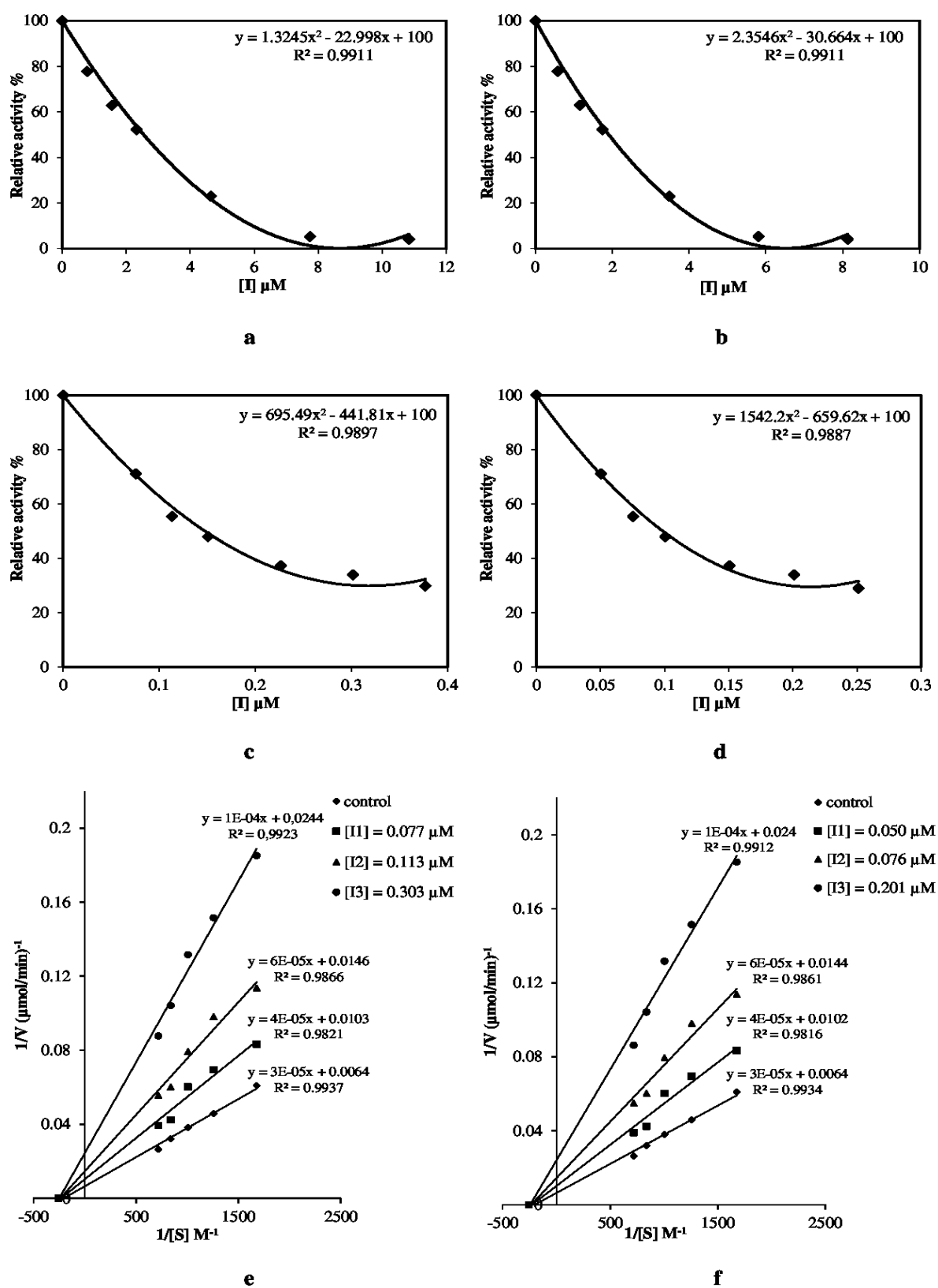


Fig. 2. Some IC_{50} and K_i plots from our study. hCA I hydratase IC_{50} plot of **3** (a), hCA II IC_{50} plot of **3** (b), hCA I esterase IC_{50} plot of **4** (c), hCA II esterase IC_{50} plot of **4** (d), hCA I Lineweaver-Burk plot of **4** (e), and hCA II Lineweaver-Burk plot of **4** (f)

Table 3

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

Compound	Hydratase IC ₅₀ ^{a,b} (μM)		Esterase IC ₅₀ ^{a,b} (μM)		K _i ^{a,b} (μM)	
	hCA I	hCA II	hCA I	hCA II	hCA I	hCA II
AAZ	0.39 ± 0.01	0.20 ± 0.01	0.42 ± 0.01	0.31 ± 0.01	0.26 ± 0.01	0.14 ± 0.01
<i>mabs</i>	–	–	3.14 ± 0.02	2.52 ± 0.09	0.89 ± 0.01	0.65 ± 0.01
<i>pabs</i>	30.44 ± 0.01	5.67 ± 0.01	28.14 ± 0.01	5.36 ± 0.01	26.32 ± 0.01	4.14 ± 0.01
mal	–	–	–	–	–	–
Clabt	–	–	850.00 ± 0.02	820.02 ± 0.01	–	–
CuClabt	–	–	290.01 ± 0.01	270.04 ± 0.01	–	–
Hmabsmal	–	–	7.11 ± 0.17	6.24 ± 0.25	3.64 ± 0.14	3.02 ± 0.17
H <p>absmal</p>	84.07 ± 0.02	56.48 ± 0.01	1.64 ± 0.01	1.51 ± 0.01	1.02 ± 0.01	0.93 ± 0.01
CuH <p>absmal</p>	13.34 ± 0.01	10.58 ± 0.01	1.43 ± 0.01	1.03 ± 0.01	0.98 ± 0.01	0.91 ± 0.01
1	–	–	8.71 ± 0.19	5.42 ± 0.14	4.25 ± 0.10	3.21 ± 0.20
2	5.04 ± 0.05	4.12 ± 0.10	0.36 ± 0.04	0.24 ± 0.02	0.15 ± 0.01	0.11 ± 0.02
3	1.09 ± 0.11	0.86 ± 0.06	1.43 ± 0.09	0.98 ± 0.07	0.86 ± 0.05	0.71 ± 0.05
4	1.68 ± 0.09	1.19 ± 0.03	0.15 ± 0.03	0.08 ± 0.01	0.06 ± 0.01	0.02 ± 0.01
5	2.33 ± 0.08	1.37 ± 0.02	4.11 ± 0.12	3.74 ± 0.16	1.92 ± 0.16	0.97 ± 0.04

^aMean ± standard error from three different assays.^b $p < 0.0001$ for all analyses.

The number of compounds inhibiting esterase activities of hCA I and II is higher than the number of compounds inhibiting hydratase activities. The fact that the *p*-nitrophenyl acetate, which is the esterase substrate, is a molecule larger than the hydratase substrate, CO₂, and it is more affected by the presence of inhibitory agents, may help explain the difference in the inhibition status. The esterase inhibition effects of the Clabt and CuClabt compounds are negligible in such a way that the K_i values could not be calculated since these compounds do not show an inhibition effect of more than 50 % required for K_i calculation. The order of esterase inhibition potential of many compounds is similar to the order of hydratase inhibition potential. However, contrary to expectations, the esterase inhibition potential of the *mabs* compound is considerably stronger than the esterase inhibition potential of the *pabs* compound. This is true only for the starting compounds, whereas for other derivatives, as expected, the inhibitory potential of the *para* derivatives is greater. The strongest inhibitor compound among the *mabs* derivatives is **3**. This can be explained by the bulky structure of the compound and the presence of various groups in its structure, as in the inhibition of hydratase activity. The inhibition potentials of the *para* derivatives increased steadily from the free ligand to the proton transfer salt complex (*pabs* < H

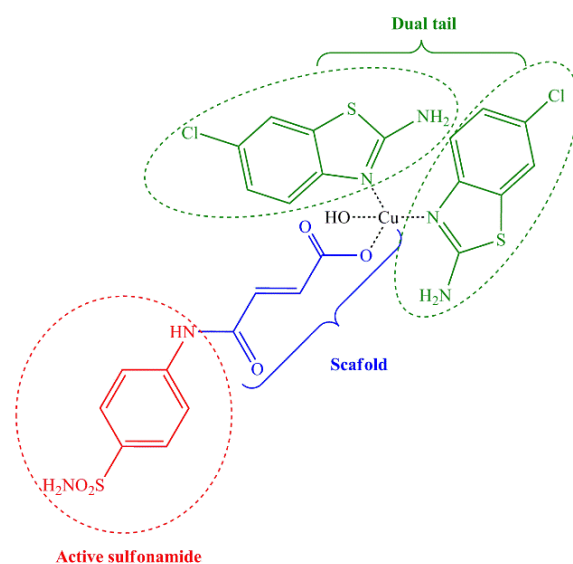
absmal

 < CuH

absmal

 < **2** < **4**). The addition of new groups to the structure increased the inhibition effect, especially in compounds **2** and **4**. Compound **4**, the

most potent esterase inhibitor in the present research, fits the "dual-tail approach" strategy already studied by some research groups.^{31,32} In this way, compounds can interact more strongly with active site amino acids (Fig. 3).

Fig. 3. Representation of compound **4** according to the dual-tail approach strategy

In addition, compound **4** shows a nearly three times stronger inhibitory effect than the control compound, **AAZ** (Table 3). The inhibition constants of the compounds (K_i values) have been determined with esterase activity method. Since

the inhibition potentials of the compounds are in the same order as the esterase inhibition potential, the structure-activity relationships in the inhibition of esterase activity are also valid in this section. The fact that the K_i values of the compounds can be determined indicates that they interact with the enzyme with sufficient strength.

In case the isoenzyme selectivity strategy mentioned in the introduction of the study is to be evaluated, it can be said that compound **4** is relatively successful in this respect. This compound inhibited the hCA II isoenzyme approximately three times stronger than the hCA I isoenzyme.

4. CONCLUSIONS

In the present work, two new salts (**1** and **2**) of sulfamoylphenylmaleamide acid and three Cu(II) complexes (**3–5**) were prepared for the first time. The structures of **1–5** are explained by the results of the spectroscopic methods.

All compounds have potential inhibition of the esterase activities of hCA I and hCA II isoenzymes. As seen from the K_i values, especially compounds **2–4** along with H_pabsmal and CuH_pabsmal show remarkable inhibition effects. In addition, compound **4** reveals more potential than other compounds in terms of isoenzyme selectivity. From the results of this study, it can be seen that the isoenzyme selectivity can be increased by using the dual-tail approach strategy and making some modifications to the existing compounds. The compounds synthesized in our research should help and assist future studies in this respect.

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Conflict of interest. The authors declare that they have no conflict of interest.

REFERENCES

- (1) Jonckers, T. H. M.; Rouan, M. C.; Haché, G.; Schepens, W.; Hallenberger, S.; Baumeister, J.; Sasaki, J. C., Benzoxazole and benzothiazole amides as novel pharmacokinetic enhancers of HIV protease inhibitors. *Bioorg. Med. Chem. Lett.* **2012**, *22* (15), 4998–5002. <https://doi.org/10.1016/j.bmcl.2012.06.022>
- (2) Supuran, C. T.; Casini, A.; Mastrolorenzo, A.; Scozzafava, A., COX-2 selective inhibitors, carbonic anhydrase inhibition and anticancer properties of sulfonamides belonging to this class of pharmacological agents. *Mini-Rev. Med. Chem.* **2004**, *4*(6), 625–632. <https://doi.org/10.2174/1389557043403792>
- (3) Masini, E.; Carta, F.; Scozzafava, A.; Supuran, C. T., Antiglaucoma carbonic anhydrase inhibitors: a patent review. *Exp. Opin. Therap. Pat.* **2013**, *23*(6), 705–716. <https://doi.org/10.1517/13543776.2013.794788>
- (4) Chohan, Z. H.; Hassan, M.; Khan, M. K.; Supuran, C. T., *In vitro* antibacterial, antifungal and cytotoxic properties of sulfonamide-derived Schiff's bases and their metal complexes. *J. Enz. Inh. Med. Chem.* **2005**, *20*(2), 183–188. <https://doi.org/10.1080/14756360500043257>
- (5) Scozzafava, A.; Owa, T.; Mastrolorenzo, A.; Supuran, C. T., Anticancer and antiviral sulfonamides. *Curr. Med. Chem.* **2003**, *10*(11), 925–953. <https://doi.org/10.2174/0929867033457647>
- (6) Sentürk, M.; Alici, H. A.; Beydemir, Ş.; Küfrevioğlu, Ö. İ., *In vitro* and *in vivo* effects of some benzodiazepine drugs on human and rabbit erythrocyte carbonic anhydrase enzymes. *J. Enz. Inh. Med. Chem.* **2012**, *27*(5), 680–684. <https://doi.org/10.3109/14756366.2011.607447>
- (7) Imran, S.; Taha, M.; Ismail, N. H.; Fayyaz, S.; Khan, K. M.; Choudhary, M. I., Synthesis of novel bisindolylmethanes: New carbonic anhydrase II inhibitors: docking and 3D pharmacophore studies. *Bioorg. Chem.* **2016**, *68*, 90–104. <https://doi.org/10.1016/j.bioorg.2016.07.011>
- (8) Bulli, I.; Dettori, I.; Coppi, E.; Cherchi, F.; Venturini, M.; Di Cesare Manelli, L.; Ghelardini, C.; Nocentini, A.; Supuran, C. T.; Pugliese, A. M.; Pedata, F., Role of carbonic anhydrase in cerebral ischemia and carbonic anhydrase inhibitors as putative protective agents. *Inter. J. Mol. Sci.* **2021**, *22*(9), 5029. <https://doi.org/10.3390/ijms22095029>
- (9) Supuran, C. T., Structure and function of carbonic anhydrases. *Biochem. J.* **2016**, *473*(14), 2023–2032. <https://doi.org/10.1042/BCJ20160115>
- (10) Sarikaya, B.; Ceruso, M.; Carta, F.; Supuran, C. T., Inhibition of carbonic anhydrase isoforms I; II, IX and XII with novel Schiff bases, identification of selective inhibitors for the tumor-associated isoforms over the cytosolic ones. *Bioorg. Med. Chem.* **2014**, *22*(21), 5883–5890. <https://doi.org/10.1016/j.bmc.2014.09.021>
- (11) Gao, B. B.; Clermont, A.; Rook, S.; Fonda, S. J.; Srinivasan, V. J.; Wojtkowski, M.; Fujimoto, J. G.; Avery, R. L.; Arrigg, P. G.; Bursell, S. E.; Aiello, L. P.; Feener, E. P., Extracellular carbonic anhydrase mediates hemorrhagic retinal and cerebral vascular permeability through prekallikrein activation. *Nat. Med.* **2007**, *13*, 181–188. <https://doi.org/10.1038/nm1534>
- (12) Kumar, R.; Kumar, A.; Ram, S.; Angeli, A.; Bonardi, A.; Nocentini, A.; Gratteri, P.; Supuran, C. T.; Sharma, P. K., Novel benzenesulfonamide-bearing pyrazoles and 1,2,4-thiadiazoles as selective carbonic anhydrase inhibitors. *Arc. der Phar.* **2022**, *355*, e2100241. <https://doi.org/10.1002/ardp.202100241>
- (13) Pacchiano, F.; Carta, F.; McDonald, P. C.; Lou, Y.; Vullo, D.; Scozzafava, A.; Dedhar, S.; Supuran, C. T., Ureido-substituted benzenesulfonamides potently inhibit carbonic anhydrase IX and show antimetastatic activity in a model of breast cancer metastasis. *J. Med. Chem.* **2011**, *54*(6), 1896–1902. <https://doi.org/10.1021/jm101541x>
- (14) Téllez, F.; López-Sandoval, H.; Castillo-Blum, S. E.; Barba-Behrens, N., Coordination behavior of

- benzimidazole, 2-substituted benzimidazoles and benzothiazoles towards transition metal ions. *Arkivoc* **2008**, (v), 245–275.
- (15) İlkimen, H.; Tekşen, Y.; Yenikaya, C.; Turhan, İ.; Tunç, T.; Sarı, M., Synthesis, characterization and pharmacological evaluation of the proton transfer salts of 2-aminobenzothiazole derivatives with 5-sulfosalicylic acid and their Cu(II) complexes. *J. Coord. Chem.* **2018**, *71*(16–18), 2831–2842. <https://doi.org/10.1080/00958972.2018.1504035>
- (16) İlkimen, H.; Yenikaya, C.; Sarı, M.; Bülbül, M.; Tunca, E.; Dal, H., Synthesis and characterization of a proton transfer salt between 2,6-pyridinedicarboxylic acid and 2-aminobenzothiazole and its complexes and their inhibition studies on carbonic anhydrase isoenzymes. *J. Enz. Inh. Med Chem.* **2014**, *29*(3), 353–361. <https://doi.org/10.3109/14756366.2013.782299>
- (17) İlkimen, H.; Yenikaya, C.; Bülbül, M.; İmdat, G., Synthesis and characterization of proton transfer salt between maleamic acid derivative including sulfonamide moiety and 2-aminopyridine and preparation of their Co(II) and Cu(II) complexes and investigation of inhibition properties on carbonic anhydrase isoenzymes. *Celal Bayar Univ. J. Sci.* **2017**, *13*(1), 211–225. <https://doi.org/10.18466/cbayarfb.302655>
- (18) Yenikaya, C.; İlkimen, H.; Demirel, M. M.; Ceyhan, B.; Bülbül, M.; Tunca, E., Preparation of two maleic acid sulfonamide salts and their Cu(II) complexes and antiglaucoma activity studies. *J. Braz. Chem. Soc.* **2016**, *27*(10), 1706–1714. <https://doi.org/10.5935/0103-5053.20160051>
- (19) Yenikaya, C.; İlkimen, H.; Ceyhan, B.; Demirel, M. M.; Tunca, E.; Bülbül, M., Synthesis and structural studies of proton transfer salt between benzimidazole and (*E*)-3-(4-sulfamoylphenylcarbonyl)acrylic acid and their transition metal complexes, and investigation of their inhibition properties. *Sakarya Univ. J. Sci.* **2017**, *21*(3), 454–462. <https://doi.org/10.16984/saufenbilder.221276>
- (20) Kremlev, M. M.; Kul'chitskaya, N. E.; Biba, A. D.; Romanenko, V. D., Arenesulfonamides. XXVII. N-(sulfamoylaryl)maleimides. *Khim. Tekh.* **1971**, *21*, 5–10.
- (21) Bergmann, F.; Schapiro, D., Further acylation experiments with sulfanilamide and heterocyclic amines. *J. Org. Chem.* **1942**, *7*(5), 419–423. <https://doi.org/10.1021/jo01199a004>
- (22) Misra, R. P.; Mahapatra, B. B.; Guru, S., Cobalt(II) and copper(II) complexes with substituted aminobenzothiazoles. Part II. *Journal of the Indian Chemical Soc.* **1981**, *58*(8), 808–810.
- (23) Rickli, E. E.; Ghazanfar, S. A.; Gibbson, B. H.; Edsall, J. T., Carbonic anhydrases from human erythrocytes: Preparation and properties of two enzymes. *J. Bio. Chem.* **1964**, *239*(4), 1065–1078. [https://doi.org/10.1016/S0021-9258\(18\)91392-X](https://doi.org/10.1016/S0021-9258(18)91392-X)
- (24) Bradford, M. M., A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–254. [https://doi.org/10.1016/0003-2697\(76\)90527-3](https://doi.org/10.1016/0003-2697(76)90527-3)
- (25) Laemmli, U. K., Cleavage of structural proteins during the assembly of the head of Bacteriophage T4. *Nature* **1970**, *22*, 680–685. <https://doi.org/10.1038/227680a0>
- (26) Wilbur, K. M.; Anderson, N. G., Electrometric and colorimetric determination of carbonic anhydrase. *J. Bio. Chem.* **1948**, *176*, 147–154. [https://doi.org/10.1016/S0021-9258\(18\)51011-5](https://doi.org/10.1016/S0021-9258(18)51011-5)
- (27) Verpoorte, J. A.; Mehta, S.; Edsall, J. T. Esterase activities of human carbonic anhydrases B and C. *J. Bio. Chem.* **1967**, *242*, 4221–4229. [https://doi.org/10.1016/S0021-9258\(18\)95800-X](https://doi.org/10.1016/S0021-9258(18)95800-X)
- (28) Lineweaver, H.; Burk, D., The determination of enzyme dissociation constants. *J. Am. Chem. Soc.* **1934**, *56*(3), 658–666. <https://doi.org/10.1021/ja01318a036>
- (29) Geary, W. J., The use of conductivity measurements in organic solvents for the characterisation of coordination compounds. *Coord. Chem. Rev.* **1971**, *7*, 81–121. [https://doi.org/10.1016/S0010-8545\(00\)80009-0](https://doi.org/10.1016/S0010-8545(00)80009-0)
- (30) Alterio, V.; Di Fiore, A.; D'Ambrosio, K.; Supuran, C. T.; De Simone, G., Multiple binding modes of inhibitors to carbonic anhydrase: How to design specific drugs targeting 15 different isoforms? *Chem. Rev.* **2012**, *112*, 4421–4468. <https://doi.org/10.1021/cr200176r>
- (31) Tanpure, R. P.; Ren, B.; Peat, T. S.; Bornaghi, R. F.; Vullo, D.; Supuran, C. T.; Poulsen, S. A., Carbonic anhydrase inhibitors with dual-tail moieties to match the hydrophobic and hydrophilic halves of the carbonic anhydrase active site. *J. Med. Chem.* **2015**, *58*(3), 1494–1501. <https://doi.org/10.1021/jm501798g>
- (32) Tawfik, H. O.; Petreni, A.; Supuran, C. T.; El-Hamamsy, M. H., Discovery of new carbonic anhydrase IX inhibitors as anticancer agents by toning the hydrophobic and hydrophilic rims of the active site to encounter the dual-tail approach. *Euro. J. Med. Chem.* **2022**, *232*, 114190. <https://doi.org/10.1016/j.ejmech.2022.114190>