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Synthesis and Biological Evaluation of Carbonic Anhydrase III and IX Inhibitors using Gas Chromatography with Modified pH-Sensitive Pellets

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ABSTRACT

Fifteen compounds were synthesized and tested as potential carbonic anhydrase III (CAIII) and carbonic anhydrase IX (CAIX) inhibitors, six of which are novel. Amides (**a1-4**), hydroxamic acids (**b1-2**), and imines (**c1-9**) derivatives were evaluated for their inhibitory activity against CAII and CAIX using gas chromatography with modified pH-sensitive pellets. The derivatives showed inhibition percentages between 12-56% for CAIII and 44-59% for CAIX, compared to 49% and 63% for captopril (the positive control), respectively. Imines showed the best inhibition of CAIII, while all derivatives showed comparable activity against CAIX. It is hypothesized that the nitrogen atom in imine, amide, or hydroxamic acid moieties in the vicinity of an ionizable group is in coordination with the zinc ion in the active site. Furthermore, the candidates were tested for their antimicrobial and antifungal activity. Generally, they showed low to zero activity against some gram-positive and negative bacteria. This supports the theory of their ability to bind to human carbonic anhydrase but not to bacterial one. These compounds could serve as useful scaffolds to develop more potent and selective carbonic anhydrase inhibitors as anti-obesity and anticancer candidates.

Keywords: Carbonic anhydrase III, Carbonic anhydrase IX, inhibitors, amides, hydroxamic acids, imines, zinc chelation.

Introduction

Carbonic anhydrases (CAs) are metalloenzymes spread in archaea, bacteria, plants, and eukaryotes[1, 2] Up to date, there are eight distinct classes of CAs α, β, γ, δ, η, ζ, and ι [1]. CAs catalyze the reaction involving the conversion of carbon dioxide to bicarbonate and protons, which is essential for pH regulation, electrolytes, gases, and anion balance[1,3]. Furthermore, CAs exhibit weak esterase activity[4].

The classes (α, β, γ, and ι) are the ones that exist in bacteria[5], β-CAs are the most distributed class in bacteria[6], where they are required for CO_2 transport[7, 8], and the growth of the microbe in general[5]. α and β CAs are abundant in fungi and yeasts, which have a crucial role in the fungi's survival. Sulfonamides, dithiocarbamates, and thiols were reported to show activity on fungi such as *candida albican* [9].

α-CA isoform is expressed in human cells and has a significant role in human pathology[4]. Human CA inhibitors such as sulfonamides have been in clinical use for decades as diuretics, anti-obesity, antiglaucoma, antiepileptic, and even anti-cancer[1, 10], [11-13]. Twelve isoforms out of fifteen known human CAs are catalytically active[3].

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Carbonic anhydrase III (CAIII), which belongs to α-CAs, is considered one of the weakest carbonic anhydrases regarding its catalytic activity in converting CO_2 to carbonic acid [3, 13, 14]. It has also shown weak binding with sulfonamides, the classical CAs inhibitors[13, 14]. Regulation of adipogenesis is one of the proposed physiological roles for CAIII. In addition to its high expression in adipocytes[3], Mitterberger *et al.* figured out that CAIII knocked-down adipocytes are associated with gene expressions leading to altering lipid metabolism[15], making CAIII a reasonable candidate for agents targeting hyperlipidemia[10, 16, 17].

On the other hand, Carbonic anhydrase IX (CAIX) is a tumor-associated isoform, significantly expressed in a wide variety of solid tumors[2]. Its expression is associated with hypoxic cancer types [3]. Its contribution to pH regulation helps protect the tumor cell from acidosis and contributes to the chemoresistance of some basic anticancer agents [18-20].

Up to date, several classes of CAs inhibitors are known[21, 22]. Metal complexing anions[1], unsubstituted sulfonamide and sulfamate[6, 21, 22], sulfamic acid, phenylboronic acid [23], dithiocarbamates[5], carboxylic acids [5], sulfonamides and their derivatives have been the most class investigated[21, 22]. Sulfa drugs are an old class of antibacterial agents. However, sulfonamides have many other pharmacological actions rather than antimicrobial such as anti-diabetes, antifungal, antimalarial, diuretics (e.g., hydrochlorothiazide), Alzheimer's disease [24] and rheumatoid arthritis (Sulfasalazine)[9]. This extended targets of sulfonamides in addition to their ability to inhibit both the human and bacterial carbonic anhydrase, such as ethoxzolamide and acetazolamide which were found to inhibit *Helicobacter Pylori* and vancomycin-resistant *Enterococcus spp*. respectively[5], rising up more side effects and make sulfonamides not ideal CAs inhibitor [10]. This encourages the rationale of finding new classes rather than sulfonamide to inhibit carbonic anhydrase. Accordingly, previous projects conducted by our team to find out new selective agents including vanillic acid, benzoic acid and nicotinic acid derivatives [10, 16, 17] where it was revealed the importance of carboxylic acid moiety in coordinating to Zn atom. In this study a group of variant moieties were synthesized and biologically tested against CAIII and CAIX.

2. Results and Discussion

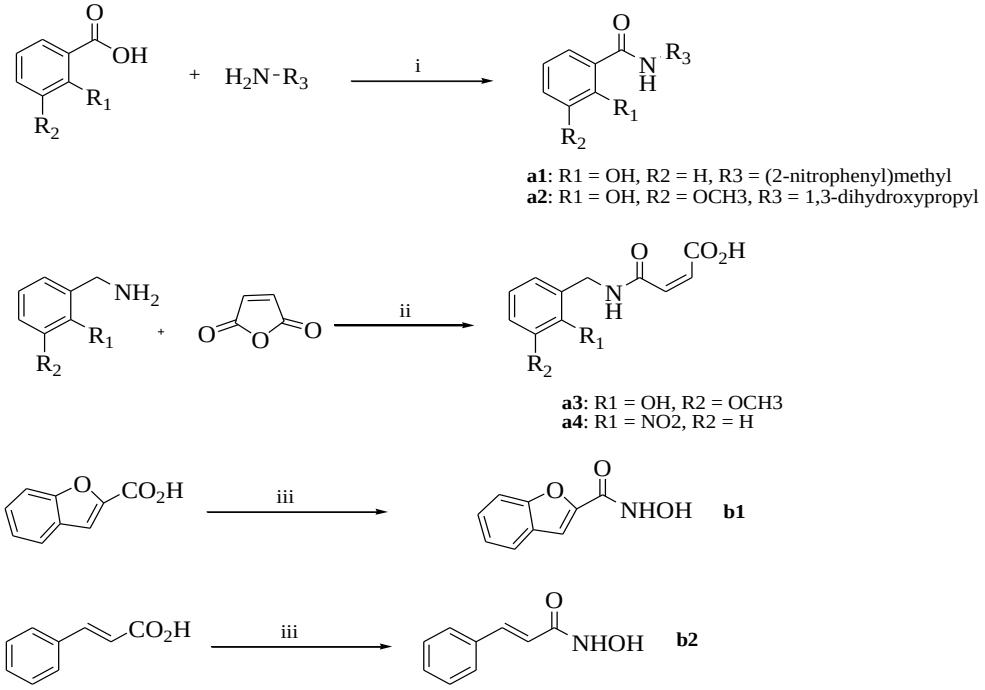
2.1 Synthesis

Based on the fact that the zinc atom is responsible for the binding mode of the CAs enzymes to their inhibitors[25], this study focuses on finding new zinc binder moieties rather than sulfonamide and testing them as potential CAIII and CAIX inhibitors. Amide, imine, and hydroxamic acid derivatives were synthesized and biologically tested[26-28].

As shown in **scheme** (**1**), amide derivatives (**a1-2**) were synthesized by the direct amidation reaction between the carboxylic acid and the amines. The carbonyl group is activated using DCC, and HOBT catalyzes the reaction[29-31]. The synthesis of amides (**a3-4**) was performed by the direct nucleophilic addition reaction of the corresponding amine with maleic anhydride[32, 33].

Hydroxamic acid derivatives (**b1-2**) [34, 35] were synthesized by converting the corresponding carboxylic acid into the acid chloride using oxalyl chloride, then treating the crude acid chloride with hydroxylamine hydrochloride[36].

Imines (Schiff bases; **c1-c9**), shown in **scheme (2),** were prepared by the classical nucleophilic addition reaction of a 1^{o} amine with an aldehyde[36]. *o*-Vanillin, vanillin, and quinoline-8-carbaldehyde were used to synthesize imines **c1**, **c2**[37], **c3**[38], **c4**[39], **c5**[40], **c6**[41], **c7**[42], **c8**, and **c9**[43].

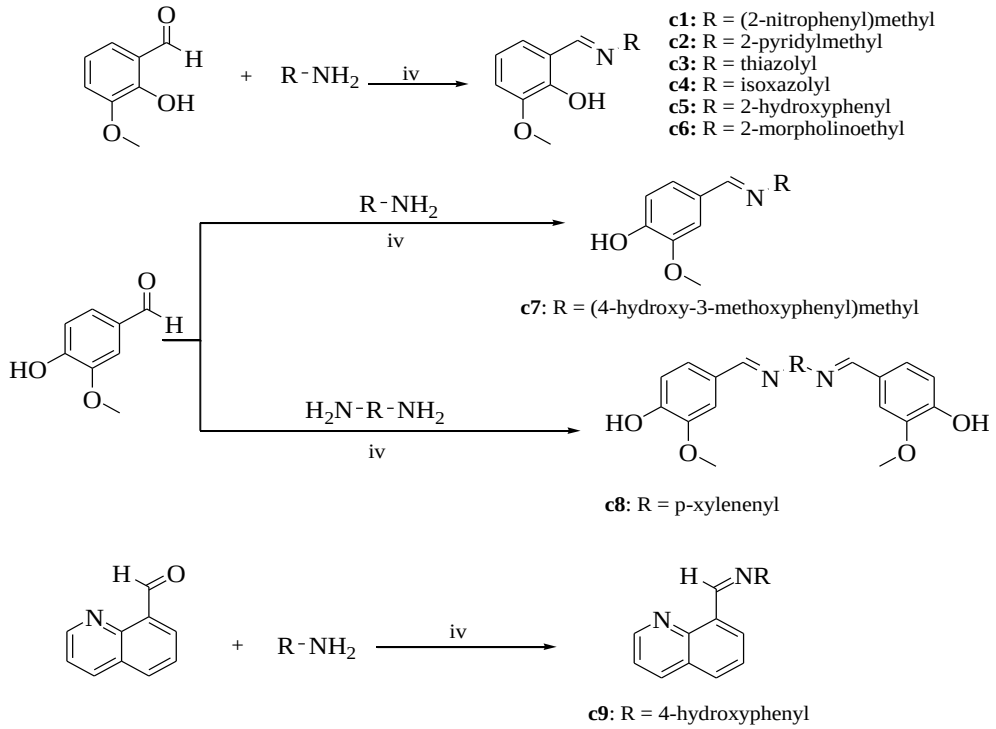


a1: R1 = OH, R2 = H, R3 = (2-nitrophenyl)methyl
a2: R1 = OH, R2 = OCH3, R3 = 1,3-dihydroxypropyl

a3: R1 = OH, R2 = OCH3
a4: R1 = NO2, R2 = H

b1 **b2**

Scheme 1: Chemical synthesis of the different amide and hydroxamic acid derivatives: i) DCC, HOBt/THF, stir, r.t, ii) 1) TEA, MeOH, rt 2) 1M HCl iii) 1) oxalyl chloride, DMF, DCM 2) $NH_2OH.HCl$, KOAc, rt



c1: R = (2-nitrophenyl)methyl
c2: R = 2-pyridylmethyl
c3: R = thiazolyl
c4: R = isoxazolyl
c5: R = 2-hydroxyphenyl
c6: R = 2-morpholinoethyl

c7: R = (4-hydroxy-3-methoxyphenyl)methyl

c8: R = p-xylenenyl

c9: R = 4-hydroxyphenyl

Scheme 2: Chemical synthesis of the different imine derivatives: iv) CH_3OH or CH_3CH_2OH

2.2 Inhibition of Enzyme Activity

Fifteen compounds were screened for their inhibitory activity against CAIII and CAIX using the new method adopted by our team. Alzweiri et al. presented a novel gas chromatography-based approach with good GC-FID detection sensitivity[44, 45]. The approach is based on using pH-sensitive pellets to assess CA esterase activity. These Pellets comprise a volatile chemical (limonene) encapsulated in a calcium alginate matrix and a pH-sensitive polymer such as eudragit E. As the medium becomes more acidic, reaching pH 5, the action enzyme activity increases the emission of the volatile chemical limonene. The suggested inhibitor will lower the enzyme's activity and, therefore, release limonene. The fluctuation in the amount of the volatile chemical reflects the tested drug's action against CAs[17]. Captopril was employed as a positive control because, as previously documented by our research, it has strong action against CA[4].

Table 1: Inhibition percentage of the proposed inhibitors against CAIII and CAIV

	CAIII Inhibition% (1 µM)	**CAIX Inhibition% (1 µM)**
a1	46.1	58.35
a2	45.02	51.48
a3	17.49	46.12
a4	31.08	44.79
b1	24.38	59.64
b2	not tested	not tested
c1	56.6	54.87
c2	24.0	48.28
c3	31.36	55.93
c4	26.23	54.59
c5	14.8	55.14
c6	44.42	49.61
c7	47.18	54.19
c8	12.3	51.29
c9	42.87	53.55
Captopril	49.38	63.52

Table (1) shows the inhibition percentage of the synthesized derivatives. The imine **c1** shows the best inhibition percentage against CAIII (56.6%) at 1 µM, whereas imine derivative **c2** showed a percent inhibition of only 24%, and that of **c5** dropped to 14.8%. Similarly, the percentage inhibition of **a4** (31.08%) is about twice as much as that of **a3** (17.49%). This variation in the inhibitory action could be attributed to the existence of the highly polar nitro group in close vicinity to the nitrogen atom of either the imine or the amide, which in turn may allow for the chelation of the zinc ion.

Looking at the imine derivative **c6** with a percentage inhibition of 44.42%, this could be attributed to a similar analogy that the nitrogen atom of the morpholine ring could be positioned in close vicinity to the imine nitrogen through rotation around the carbon-carbon single bond in an eclipsed conformation, allowing once more for the chelation to the zinc ion.

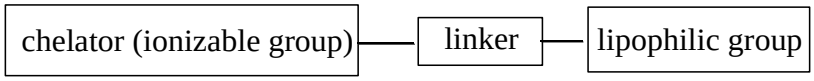
The close vicinity between the imine nitrogen and the quinolone nitrogen in derivative **c9**, allowing for the chelation

to the zinc ion, may also explain the somewhat high percent inhibition of this derivative with 42.87%.

The importance of the vicinity of two chelating groups for the successful binding to the zinc ion may be exemplified by the low percentage inhibition (12.3%) of the imine derivative **c8**. In this derivative, there is no close donor atom to the imine nitrogen, thus limiting the possible chelation to the zinc ion, which needs a bidentate ligand for chelation.

This hypothesis could be extended to explain the percent inhibition of **a1** (46.1%) and **a4** (31.08%). Both derivatives have a nitro group in the vicinity of the amide nitrogen, forming a bidentate suitable to bind to the zinc ion. Regarding the amide derivative **a2**, with a percent inhibition of 45.02%, the free rotation around the carbon-carbon single bond of the primary alcohol brings the donor oxygen atom in close vicinity to the amide nitrogen, allowing for the chelation of the zinc ion.

For CAIX, the percentage inhibition, in general, was higher than for CAIII with almost all the derivatives. The inhibition percentages were around (40-60)%. Captopril has higher % inhibition; 49.38% for CAIII and 63.52% for CAIX. This could be explained by the difference in the active site where the CAIII is narrow and cone-like and has less catalytic activity than other CAs. CAIX has a hydrophobic region about 10-15 A° away from the zinc atom and is close to the entrance of the active site[46]. These differences in the size and shape of the active site may rely on the differences in the activity and the ability of the CAIX to interact with the different scaffolds used in this project. The proposed SAR could be summarized as in **scheme (3)** below:



Scheme 3: Summary of the proposed SAR of the tested compounds as CA inhibitors

The chelator could be varied, such as the imine and the adjacent nitro group, or the imine and the adjacent amine group, or the amide and the adjacent nitro group, or even the amide with the adjacent hydroxyl group. These could be seen in **c1**, **c6**, **a1**, and **a2,** respectively. At the same time, the lipophilic part of the derivatives could be much more important for the CAIX than for CAIII as the pocket is small and narrow. Consequently, not all the derivatives show comparable inhibition percentage for both enzymes. The lipophilic part varied between the benzene ring, benzofuran (**b1**), and quinoline (**c9**).

In general, the inhibition percentages reported in this work show a good ability to inhibit CAIII and CAIX with comparable results to the captopril (the positive control) and some reported results by our team. For example, nicotinic acid derivatives which showed oxygen coordinate to the zinc atom gave inhibition percentage range between 40-90 % at 10 µM[45].

2.3 Antimicrobial and Antifungal Activity

The previous results encouraged testing some synthesized compounds against their ability to inhibit bacterial growth. Bearing in mind that β-CAs active site poses little similarity with human α-CAs increasing the possibility of finding selective α-CAs inhibitors[8]. The synthesized compounds were tested to find their antimicrobial activity. Kirby-Bauer Disk, Diffusion Susceptibility Test was performed to know the inhibition zone for the samples. While the Broth dilution method (196 microplates well) was used to determine the lowest concentration of an antimicrobial agent that prevents visible growth of the microorganism, the result was calculated by calculating the mean value. **Table** (**2**) lists the derivatives that showed activity against different bacterial strains and *Candida albicans*. Imine derivatives (**c1-4**) were inactive against bacterial strain but showed good activity against *candida albicans* with MIC ranging from (0.625-1.25 mM) compared to 0.15 mM for Nestatine as a control.

Meanwhile, hydroxamic acid derivatives (**b1-2**) showed antimicrobial activity with a low millimolar compared to the control, while **b1** showed activity against *C. albicans* with equal MIC to that of imines. Unfortunately, **b2** did not show the same activity. The amide derivatives (**a1-4**) were inactive at these concentrations. It is known that hydroxamic acids have good antimicrobial, antifungal and antitumor activity owing to their ability to bind to many metalloenzymes. They also have low toxicity and weak acid properties[47-49]. These results support the hypothesis of the ability of these agents to inhibit CAs via zinc complexation. Thus, the track will keep open for further modification on these derivatives to enhance their activity.

Table 2: Antimicrobial activity by disc diffusion method (mm) and Minimum inhibition concentration (MIC) for the tested compounds against different organisms.

	Disc Diffusion method (mm)			Minimum Inhibition Concentration (MIC)		
Tested compound	***Escherichia coli*** **(mm)**	***Staphylococcus aureus*** **(mm)**	***Candida albicans*** **(mm)**	***Escherichia coli*** **(mM)**	***Staphylococcus aureus*** **(mM)**	***Candida albicans*** **(mM)**
c1	0	0	16	-----	-----	1.25
c2	0	0	15	-----	-----	0.625
c3	0	0	16	-----	-----	1.25
c4	0	0	16	-----	-----	1.25
b1	12	8	19	1.25	2.5	1.25
b2	0	8	0	------	2.5	-------
Levofloxacin	24	32	0	0.00488	0.025	-------
Amoxicillin/ Clavulanic acid	20	42	0			
Trimethoprim/ Sulfamethoxazol	24	21	0			
Nystatin	0	0	21	------	------	0.15

The synthesized compounds showed inactivity to low activity against some gram-positive and negative bacteria, which is considered good as we propose their ability to inhibit the human carbonic anhydrases rather than the bacterial ones.

3. Experimental

3.1. Synthesis

3.1.1. General procedure for the synthesis of amide derivatives (a1-2)

After adding the amine to a solution of the carboxylic acid (1 mmoL) in dry THF (15 mL) and stirring, the DCC (1.2 mmoL) and HOBT (1.2 mmoL) were added (1 mmoL). Overnight, the reaction mixture was left to stir at room temperature. Ethyl acetate was extracted from the white precipitate and diluted with water (30 mL) (x3). After brine washing, drying ($MgSO_4$), and rotational evaporation, the combined organic extracts were ready for use. The amide product crystallized out of the ethyl acetate/hexane mixture and appeared white.

N-(2-Nitrobenzyl)-2-hydroxybenzamide (a1)

It was collected as a white solid (0.56 g, 57%), m.p. 110-112°C. ^{1}H NMR (400 MHz, DMS-d_6): δ 12.10 (1H, s, OH), 9.35 (1H, s, NH), 8.06-8.08 (1H, d), 7.90-7.92 (1H, d), 7.73-7.75 (1H, m), 7.57-7.62 (2H, m), 7.43 (1H, m), 6.93-6.95 (2H, m), 4.81 (2H, s, CH_2N). HRMS (ESI): m/z calculated for $C_{14}H_{12}N_2O_4$ + H^+ 273.2641; found 273.0763.

N-(1,3-Dihydroxypropan-2-yl)-4-hydroxy-3-methoxybenzamide (a2)

Collected as white solid (0.18 g, 24%), m.p. 133-136°C.^{1}H NMR (400 MHz, DMS-d_6): δ 9.43 (1H, br s, OH), 7.62-7.65 (1H, d), 7.35 (1H, s), 7.28-7.30 (1H, d, NH), 6.71-6.73 (1H, d), 4.55-4.58 (2H, t, 2 x CH_2OH), 3.81-3.88 (1H, m, NHC*H*), 3.74 (3H, s, OCH_3), 3.43-3.49 (4H, t, 2 x CH_2OH). ^{13}C NMR (100 MHz, DMS-d_6): δ 165.87, 149.29, 146.98, 125.71, 120.79, 114.62, 111.43, 60.41, 55.67, 53.68. m/z calculated for $C_{11}H_{15}NO_5$ 241.24; found 241.09.

3.1.2. General procedure for the synthesis of amide derivatives (a3-4)

The amine (1.3 mmoL) was dissolved in methanol (7 mL) and triethylamine (3 mL), and then maleic anhydride was added while the mixture was agitated (1.3 mmoL). The product precipitated after being agitated in a reaction mixture containing 10 mL of 1M HCl slowly throughout the previous night. Products were filtered, washed many times, and dried in an oven overnight.

(Z)-4-(2-Hydroxy-3-methoxybenzylamino)-4-oxobut-2-enoic acid (a3)

They were prepared as previously mentioned. However, the product was extracted after adding 1M HCl using dichloromethane. The desired chemical was obtained as a white solid (0.21g, 68%) when the mixed organic extracts were washed with water, dried (MgSO4), and evaporated.^{1}H NMR (400 MHz, DMS-d_6): *δ* 9.38-9.40 (1H, t, NH), 8.07 (1H, br s, OH), 6.73-6.91 (3H, m), 6.47-6.50 (1H, d), 6.26-6.29 (1H, d), 4.34-4.35 (2H, d, CH_2N), 3.79 (3H, s, OCH_3). HRMS (ESI): m/z calculated for $C_{12}H_{13}NO_5 - H^+$ 250.2274; found 250.0681.

(Z)-4-((2-Nitrobenzyl)amino)-4-oxobut-2-enoic acid (a4)

It was collected as a white powder (0.25 g, 77%), m.p. 142-144°C. ^{1}H NMR (400 MHz, DMS-d_6): *δ* 9.29 (1H, br s, NH), 8.06-8.08 (1H, dd), 7.72-7.76 (1H, dt), 7.64-7.66 (1H, d), 7.55-7.59 (1H, dt), 6.43-6.46 (1H, d), 6.24-6.27 (1H, d), 4.66-4.67 (2H, d, CH_2N). ^{13}C NMR (100 MHz, DMS-d_6): δ 166.66, 165.98, 148.37, 134.32, 133.56, 132.02, 131.71, 130.51, 129.06, 125.15 (chemical shift for CH_2N carbon is combined with DMS signal peaks). HRMS (ESI): m/z calculated for $C_{11}H_{10}N_2O_5 + H^+$ 251.2155; found 251.0588.

*3.1.3. General procedure for the synthesis of hydroxamic acid derivatives (*b1-2)

Carboxylic acid (1 mmoL) and dichloromethane (1 mmoL) in dichloromethane (100 mL) were chilled to 0 degrees Celsius. Afterward, oxalyl chloride (2.5 mmoL) was injected gradually, sparking a rapid release of gas. After stirring for an hour, the reaction mixture was combined with a THF/water solution of hydroxylamine hydrochloride (4.0 mmoL) and triethylamine (6.0 mmoL) (50:10). The ingredients were combined and agitated for 30 minutes before being added to 2 N HCl. Extracting the product using dichloromethane (x 2), then washing the mixed organic layer with brine, drying it (MgSO4), and rotary-evaporating it. Aqueous ethanol was used to create the crystals.

N-Hydroxybenzofuran-2-carboxamide (b1)

Collected as white solid (0.16g, 58%), m.p. 150-153°C. ^{1}H NMR (400MHz, DMS-d_6) δ 11.47 (1H, br s), 9.28 (1H, br s), 7.76-7.78 (1H, d), 7.64-7.66 (1H, d), 7.44-7.49 (2H, m), 7.32-7.36 (1H, m). m/z calculated for $C_9H_7NO_3$ 177.16; found 177.04.

N-Hydroxycinnamamide (b2)

Collected as white solid (0.17g, 60%), 255°C (with decomposition). ^{1}H NMR (400MHz, DMS-d_6) δ 7.55-7.57 (2H, d), 7.40-7.46 (6H, m), 6.46-6.50 (1H, d). m/z calculated for $C_9H_9NO_2$ 163.17; found 163.06.

3.1.4. General procedure for the synthesis of imine derivatives (c1-c9)

To a stirred solution of the corresponding amine (10.6 mmoL) in ethanol or methanol (20 mL) was added the

aldehyde (9.7 mmoL). The reaction was stirred for (1-2) days, and then the yellow precipitate was filtered, washed with ethanol, and dried.

***(E)-2-((2-Nitrobenzylimino)methyl)-6-methoxyphenol* (c1)**

Potassium acetate (0.55 g, 5.60 mmoL) was added to the solution, and the product was crystallized from dichloromethane/ether to afford the product as yellow-orange crystals (0.28 g, 18%), m.p. 70-71°C. ^{1}H NMR (400 MHz, DMS-d_6): δ 13.21 (s, OH, 1H), 8.72 (s, CH=N, 1H), 8.09-8.07 (d, 1H), 7.80-7.76 (t, 1H), 7.65-7.59 (m, 2H), 7.09-7.07 (d, 2H), 6.88-6.84 (t, 1H), 5.11 (s, CH_2N, 2H), 3.79 (s, OCH_3, 3H). HRMS (ESI): m/z calculated for $C_{15}H_{14}N_2O_4$ + H^+ 287.2907; found 287.0912.

***(E)-2-Methoxy-6-((pyridin-2-ylmethyl)imino)methyl)phenol* (c2)**

Collected as yellow solid (0.56 g, 24%), m.p. 85-87°C. ^{1}H NMR (400 MHz, DMS-d_6): δ 13.60 (1H, br s, OH), 8.73 (1H, s, CH=N), 8.55-8.57 (dd, 1H), 7.80-7.84 (1H, dt), 7.40-7.42 (1H, d), 7.31-7.34 (1H, t), 7.05-7.09 (2H, dd), 6.81-6.85 (1H, t), 4.92 (2H, s, CH_2N), 3.78 (3H, s, OCH_3). ^{13}C NMR (100 MHz, DMS) δ 168.04, 158.17, 151.99, 149.77, 148.52, 137.52, 123.75, 123.02, 122.56, 118.92, 118.36, 115.42, 63.83, 56.22. HRMS (ESI): m/z calculated for $C_{14}H_{14}N_2O_2$ – H^+ 241.2653; found 241.0950.

***(E)-2-Methoxy-6-((thiazol-2-ylimino)methyl)phenol* (c3)**

Collected as yellow crystals (0.24 g, 39%), m.p. 91-93°C. ^{1}H NMR (400 MHz, DMS-d_6): δ 11.32 (br s, OH, 1H), 9.30-9.33 (1H, d, CH=N), 7.65-7.74 (2H, dd), 7.41-7.43 (1H, m), 7.17-7.19 (1H, m), 6.92-6.94 (1H, m), 3.83 (3H, s, OCH_3). HRMS (ESI): m/z calculated for $C_{11}H_{10}N_2O_2S$ + H^+ + H_2O 253.2986; found 253.0562.

***(E)-2-((Isoxazol-3-ylimino)methyl)-6-methoxyphenol* (c4)**

Collected as yellow solid (0.30g, 40%) m.p. 165-166°C. ^{1}H NMR (400 MHz, DMS-d_6): δ 11.90 (1H, br s, OH), 9.18-9.20 (1H, d), 8.94 (1H, s, CH=N), 7.32-7.34 (1H, m), 7.19-7.21 (1H, m), 6.94-6.98 (2H, m), 3.85 (3H, s, OCH_3). HRMS (ESI): m/z calculated for $C_{11}H_{10}N_2O_3$ + H^+ 219.2167; found 219.0751.

***(E)-2-(2-Hydroxy-3-methoxybenzylideneamino)phenol* (c5)**

Collected as orange solid (0.30g, 40%). ^{1}H NMR (400 MHz, DMS-d_6): δ 14.06 (1H, s, OH), 9.78 (1H, s, OH), 8.94 (1H, s, CH=N), 6.85-7.36 (7H, m), 3.79 (3H, s, OCH_3). m/z calculated for $C_{14}H_{13}NO_3$ 243.26; found 243.08.

***(E)-2-Methoxy-6-((2-morpholinoethylimino)methyl)phenol* (c6)**

Collected as yellow solid (0.36g, 84%). ^{1}H NMR (400 MHz, DMS-d_6): δ 13.79 (1H, br s, OH), 8.52 (1H, s, CH=N), 6.98-7.01 (2H, m), 6.74-6.78 (1H, m), 3.77 (3H, s, OCH_3), 3.69-3.72 (2H, t), 3.55-3.57 (2H, m), 3.34 (2H, m), 2.57-2.67 (2H, t), 2.43-2.50 (4H, m). m/z calculated for $C_{14}H_{20}N_2O_3$ 264.32; found 264.14.

***(E)-4-((4-Hydroxy-3-methoxybenzylimino)methyl)-2-methoxyphenol* (c7)**

Collected as yellow solid (0.26g, 73%). ^{1}H NMR (400 MHz, DMS-d_6): δ 9.53 (1H, br s, OH), 8.86 (1H, br s, OH), 8.27 (1H, s, CH=N), 7.34 (1H, m), 7.14 (1H, m), 6.69-6.86 (4H, m), 4.58 (2H, s, CH_2N), 3.78 (3H, s, OCH_3), 3.75 (3H, s, OCH_3). m/z calculated for $C_{16}H_{17}NO_4$ 287.31; found 287.12.

***4-((E)-((E)-4-(((E)-4-Hydroxy-3-methoxybenzylideneamino)methyl)benzylimino)methyl)-2-methoxyphenol* (c8)**

Collected as orange solid (0.47g, 88%). ^{1}H NMR (400 MHz, DMS-d_6): δ 13.6 (2H, br s, 2xOH), 8.90 (2H, s, 2xCH=N), 6.82-7.35 (10H, m), 4.81 (4H, s, 2xCH_2N), 3.77 (6H, s, 2xOCH_3). m/z calculated for $C_{24}H_{24}N_2O_4$ 404.46; found 404.17.

***(E)-4-(Quinolin-8-ylmethyleneamino)phenol* (c9)**

Collected as yellow solid (0.13g, 38%). ^{1}H NMR (500 MHz, DMS-d_6): δ 9.84 (1H, s, OH), 9.55 (1H, s, CH=N), 9.03 (1H, m), 8.46-8.50 (2H, dd), 8.14-8.15 (1H, d), 7.73 -7.76 (1H, t), 7.62-7.65 (1H, m), 7.26-7.28 (2H, m), 6.83-6.85 (2H, m). ^{13}C NMR (100 MHz, DMS-d_6): δ 156.9, 154.4, 151.1, 146.6, 143.7, 137.2, 133, 131.5, 128.5, 127.3, 127, 123.1, 122.4, 116.3. m/z calculated for $C_{16}H_{12}N_2O$ 248.28; found 248.09.

3.2 Inhibition of enzyme activity

Fifteen compounds were screened for their inhibitory activity against CAIII and CAIX using the method developed by Alzweiri et al. The method is based on GC-FID[45, 50]. The method utilizes pH-sensitive pellets for evaluating the esterase activity of CAs.

3.3 Antimicrobial activity

3.3.1 Materials and methods

E. coli ATCC8739, *Pseudomonas aeruginosa* ATCC9027, *Proteus mirabilis* ATCC12453, *Bacillus subtilis* ATCC6633, and *Staphylococcus aureus* ATCC6538 were utilized as bacteria, while *Candida albicans* ATCC10231 was used as yeast. All of these strains were acquired from the American Type Culture Collection. Nutrient agar (NA, Biolab, Budapest, Hungary) and Sabouraud Dextrose Agar (SDA, Biolab, Budapest, Hungary) slants were used to cultivate the bacterial and yeast strains, respectively, at 37°C.

3.3.2 Antimicrobial assay

Disc diffusion method

In vitro tests using the Kirby-Bauer agar diffusion technique determined whether or not the synthesized compounds exhibited antibacterial activity against Gram-positive and Gram-negative bacteria and Candida[51, 52]. To culture bacteria, Mueller-Hinton agar was used, and to culture yeast, Sabouraud Dextrose agar was used, as suggested by the NCCLS recommendations[53, 54].

One hundred microliters (100 µl) of microbial suspension was plated onto the Mueller-Hinton agar medium after 10 ml of new media was used to cultivate the test bacteria/Candida to a concentration of about 108 cells/ml for bacteria (0.5 McFarland standards) (MHA, oxoid, Wade Road, UK).

As previously noted, the compounds were evaluated using sterile blank discs with a diameter of 6 millimeters (antibiotic assay discs, Whatman-model 2017-006[54]. After the discs were dried, they were injected with 20 µL of 20 mM of the chemical and put on plates. Bacterial and fungal pathogens were inoculated onto plates, and after 24 hours at 37ºC, the widths of the inhibitory zones were measured. The results of each antimicrobial test were averaged from two independent runs. Positive controls for antimicrobial and Candida activity were established using standard antibiotics such as levofloxacin (5 mcg/disc), amoxicillin/clavulanic acid (30mcc), trimethoprim/sulfamethoxazole (1.25/23.75 µg), and nystatin (100mcg/disc). As a sham, we utilized discs soaked in 20 µl of DMSO. It was possible to quantify the sizes of the inhibitory zones. **Table 2** lists the synthesized compounds and their inhibitory activities against these species.

Serial dilution method (broth microdilution assay)

The MIC was calculated using the broth microdilution method, as recommended by the National Committee for Clinical Laboratory Standards [55]. After overnight incubation in broth, inocula of the bacterial strains or Candida were made, and the turbidity of the suspensions was set to 0.5 McFarland units. In a 96-well microtiter plate, the compounds were diluted serially. The diluent utilized was Mueller Hinton broth (MHA, Oxoid, Wade Road, UK). Compounds were found in quantities spanning from 2.5 to 0.00488 mM. Mueller Hinton broth was used to inoculate wells with test strains of bacteria and Candida (with final concentrations of 2.0×10^6 CFU/ml for bacteria and 2.0×10^5 CFU/ml for Candida). For 24 hours, the plate was kept at 37ºC. A well was set up with the growth media and the bacteria or Candida as a control. Positive controls included levofloxacin and

nystatin, while negative controls included DMSO. The test wells and the positive and negative controls were examined to assess the level of microbial growth. Antibacterial and antifungal actions were inferred from the lack of microbiological development. According to **Table 2**, the minimal inhibitory concentration (MIC) is defined as the lowest concentration of a substance at which observable growth of microorganisms occurs.

Conclusion

In summary, 15 compounds were synthesized and tested as CAIII and CAIX inhibitors. Compounds **c1**, **c6**, **a1**, and **a2** that have a bidentate chelating efficiency gave the best inhibition activity against CAIII, whereas all compounds had a satisfactory inhibiton activity against CAIX. Based on these results, it can be concluded that other functional groups other than the sulfonamide group could be developed as potential CAs inhibitors. It should be noted that Schiff bases synthesized in this work showed different percentage inhibition with CAIII and CAIX. As such, this may give potential to design selective inhibitors. CAs active site need further investigation, and the synthesized compounds could be used as a good starting point for other, non-sulfonamide CA inhibitors.

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Conflict of Interest

The authors confirm no conflict of interest.

Abbreviations

CA: carbonic anhydrase
CAIII: carbonic anhydrase III
CAIX: carbonic anhydrase IX
DCC: Dicyclohexylcarbodiimide
HOBT hydrate: 1-hydroxybenzotriazole hydrate
TEA: triethylamine
THF: tetrahydrofuran

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التوليف والتقييم البيولوجي لمثبطات الأنهايدريز الكربونية III و IV عن طريق كروماتوغرافيا الغاز باستخدام كريات معدلة حساسة لدرجة الحموضة

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& ساهم كل من بثينة حسين و لورانس البرغلي في هذا العمل بالتساوي.

ملخص

تم تصنيع خمسة عشر مركبًا واختبارها كمثبطات محتملة للأنهايدريز الكربوني III (CAIII) والأنهايدريز الكربوني IX (CAIX)، ستة منها مركبات جديدة. تم تقييم مشتقات الأميدات (a1-4) والأحماض الهيدروكسيمية (b1-2) والإيمينات (c1-9) لنشاطها المثبط ضد CAIII وCAIX عن طريق كروماتوجرافيا الغاز باستخدام كريات معدلة حساسة لدرجة الحموضة. أظهرت المشتقات نسب تثبيط تتراوح بين 12–56٪ لـ CAIII و44–59٪ لـ CAIX، مقارنة بـ 49٪ و63٪ لكابتوبريل (المركب المرجعي) على التوالي. أظهرت الإيمينات أفضل تثبيط لـ CAIII، بينما أظهرت جميع المشتقات نشاطًا مشابهًا ضد CAIX. من المفترض أن ذرة النيتروجين في شقوق الإيمين أو الأميد أو الحمض الهيدروكسيمي بالقرب من مجموعة قابلة للتأين بالتنسيق مع أيون الزنك في الموقع النشط. علاوة على ذلك، تم اختبار المركبات بالنسبة لنشاطهم المضاد للميكروبات والفطريات. بشكل عام، أظهرت المركبات نشاطًا منخفضًا او عدم النشاط ضد بعض البكتيريا موجبة وسالبة الجرام. هذا يدعم نظرية قدرتها على الارتباط بالأنهايدريز الكربوني البشري ولكن ليس الأنهايدريز الكربوني الخاص بالبكتيريا. يمكن أن تكون هذه المركبات بمثابة نماذج مفيدة لتطوير مثبطات الأنهايدريز الكربونية أكثر فعالية وانتقائية كمضادات للسمنة ومضادة للسرطان.

الكلمات الدالة: كربونيك أنهايدريز III، كربونيك أنهايدريز IX، مثبطات، أميدات، أحماض هيدروكسيمية، إيمينات، مخلّب الزنك.

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