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**SHORT COMMUNICATION**

# (3,4-Dihydroxyphenyl)(2,3,4-trihydroxyphenyl)methanone and its derivatives as carbonic anhydrase isoenzymes inhibitors

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**Abstract**

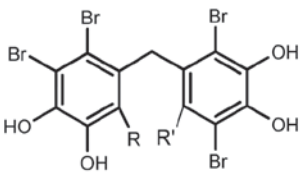
In this study, we have synthesised (3,4-dihydroxyphenyl)(2,3,4-trihydroxyphenyl)methanone and a series of its derivatives (**5**, **13–16**) and tested the ability of these compounds to inhibit two metalloenzyme human carbonic anhydrase (hCA, EC 4.2.1.1) isozymes, hCA I and hCA II. The synthesised compounds showed inhibitory effect on hCA I and hCA II isozymes. The results showed that synthesised compounds (**5, 13–16**) demonstrated the best inhibition activity against hCA I ($IC_{50}$: 3.22–54.28 μM) and hCA II ($IC_{50}$: 18.52–142.01 μM). The compound **14** showed the highest inhibiton effect against hCA I ($IC_{50}$: 3.22 μM; $K_i$: 1.19 ± 1.4 μM). On the other hand, the compound **13** showed the highest inhibiton effect against hCA II ($IC_{50}$: 18.52 μM; $K_i$: 3.25 ± 1.13 μM).

**Keywords:** Bromophenols, carbonic anhydrase, isoenzyme, enzyme inhibition

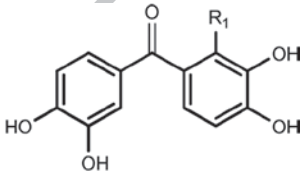


## Introduction

Bromophenols are abundantly found in marine life and they have important biological activities[1]. It was reported that some of the bromophenols (**1–3**) exhibited enzyme inhibition, cytotoxicity, feeding deterrent, antioxidant and antimicrobial activities[2]. Antioxidant[2] and carbonic anhydrase (CA) inhibitory properties[3] of the derivatives of compound **4** with Br and antioxidant properties[4] of the derivatives of compound **5** with Br were investigated.




**1** R = R' = H
**2** R = Br, R' =H
**3** R = R' = Br




**4** $R_1$ = H
**5** $R_1$ = OH

Carbonic anhydrase (CA) is a zinc metalloenzyme that catalyses the reversible reactions of $CO_2$ and water: $CO_2 + H_2O \overset{CA}{\Leftrightarrow} HCO_3^- + H^+$. It classically participates in the maintenance of pH homeostasis in human body, catalysing the reversible hydration of $CO_2$ in a two-step reaction to yield bicarbonate and protons[5,6]. CA seems to be almost ubiquitously expressed in living organisms. At least three[7] to five[8] genetically unrelated families (α, β, γ, δ and ξ) of CA isoforms exist. The α-CA family is the best studied group, although recent reviews indicate rapid advancement of knowledge about other CA families[9,10]. On the other hand, 16 isozymes have been described till now. These isoenzymes differ in their subcellular localisation, catalytic activity and susceptibility to different classes of inhibitors. Some of these isozymes are cytosolic (CA I, II, III, VII and XIII), others are membrane-bound (CA IV, IX, XII and XIV), two of them are mitochondrial (CA VA and CA VB) and other one is secreted in saliva (CA VI). It has been reported that CA XV isoform is not expressed in humans or in other primates, however, it is abundant in rodents and other higher vertebrates[11,12]. A critical problem in the design of CA inhibitors (CAIs) with pharmacological applications for the treatment and prevention of various diseases is related to the high

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number of these CA isoforms, the diffuse localisation of CAs in many tissues/organs and the lack of isozyme selectivity of the presently available inhibitors[13].

Chemicals are generally known to activate or inhibit several enzymes *in vivo* and affect metabolic pathways[14,15]. The main classes of CAIs are known: the metal complexing anions, and the substituted sulfonamides and their bioesters such as sulfamates and sulfamides, which bind to the metal ion of the enzyme either by substituting for the nonprotein zinc ligand to generate a tetrahedral adduct or by participating in the metal coordination sphere to generate trigonal bipyramidal species. Phenols are also very important inhibitors that display competitive inhibition[16–21]. The $Zn^{2+}$ ion in CAs is critical for the inhibition of these enzymes. Three mechanisms of inhibition have been proposed, one of which involves anchoring of the inhibitor to the $Zn^{2+}$-bound solvent molecule such as water or hydroxide ion. Phenols and polyamines bind in this way[12,16–21].

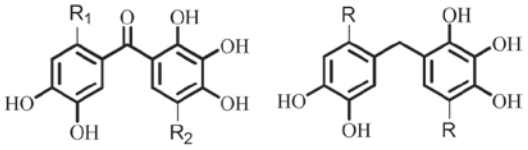
In this study, we have synthesised the novel (3,4-dihydroxyphenyl) (2,3,4-trihydroxyphenyl)methanone and a series of its derivatives (**5, 13–16**) and evaluated their potency to be novel CA isoenzymes (hCA-I and hCA-II) inhibitors.

## Results and discussion

### Synthesis

After (3,4-dimethoxyphenyl)(2,3,4-trimethoxyphenyl) methanone (**6**) was obtained from the reaction of 3,4-dimethoxybenzoic acid and 1,2,3-trimethoxybenzene in polyphosphoric acid by the known method[2,22–24], bromination reactions of **6** with different equivalents (equiv.) of ceric ammonium nitrate (CAN)/LiBr at room temperature (RT) were performed. A monobromide **7** and a dibromide **8** were obtained from these reactions as the sole products (Scheme 1)[24].

Wolff–Kishner reduction of ketone **6** gave diarylmethane derivative **9** in high yield (Scheme 1). Bromination of **9** with 1.1 equivalents of LiBr/CAN gave a mixture of three products, while its reaction with 2.2 equivalents of LiBr/CAN gave a product as sole product. From these isolated products, the product produced in the reaction as sole product was dibromide **12**, and the products found in the mixture were bromides **10**, **11** and **12** (Scheme 1)[4].



**13** $R_1$ = H, $R_2$ = Br
**14** $R_1$ = $R_2$ = Br
**15** R = H
**16** R = Br

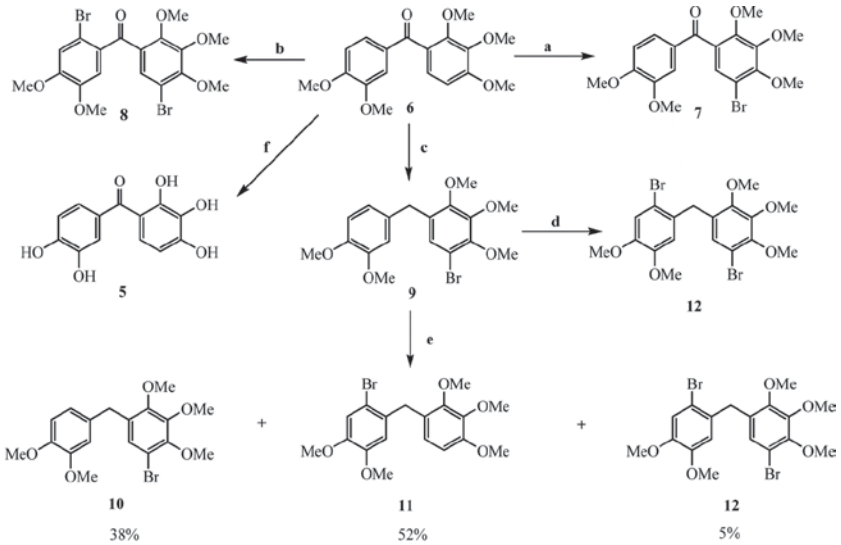
Demethylation reaction of **6** with $BBr_3$ in $CH_2Cl_2$ at 0°C–25°C gave the phenol **5** (Scheme 1). Demethylation reactions of **7–9** and **12** with $BBr_3$ in $CH_2Cl_2$ were similarly performed at 0°C–25°C. Bromophenol derivatives **13–16** were synthesised from these demethylation reactions[4].

### CA purification and assay

In this study, CA isoenzymes I and II (hCA I and hCA II) were purified from human erythrocytes (Table 1). The inhibitory effects of bromophenols were tested *in vitro*. The inhibitor concentrations that caused 50% inhibition ($IC_{50}$) were determined from activity versus (%)-[bromophenols] plots, and the $K_i$ values were calculated from Lineweaver–Burk plots (Table 1).

### Inhibition effects of CA isoenzymes

CA isoenzymes inhibitory effects of the synthesised (3,4-dihydroxyphenyl)(2,3,4-trihydroxyphenyl)methanone and a series of its derivatives (**5**, **13–16**) were tested



Scheme 1. Reagents and conditions. (a) LiBr (1.1 equiv.)/CAN (1.1 equiv.), $CH_3CN$, RT, 3 d, 97%; (b) LiBr (6.0 equiv.)/CAN (6.0 equiv.), $CH_3CN$, RT, 6 d, 95%; (c) KOH-$NH_2NH_2$/$(OHCH_2)_2$, 110°C–190°C, 3 d, 83%; (d) LiBr (2.1 equiv.)/CAN (2.1 equiv.), $CH_3CN$, RT, 3 d, 95%; (e) LiBr (1.1 equiv.)/CAN (1.1 equiv.), $CH_3CN$, RT, 3 d; (f) $BBr_3$, $CH_2Cl_2$, 96%.

Table 1. Summary of purification procedure for human carbonic anhydrase isoenzymes (hCA I and hCA II) by a sepharose-4B-thyrosine-sulfanilamide affinity column chromatography.

| Purification steps | | Activity (EU/mL) | Total volume (mL) | Protein (mg/mL) | Total protein (mg) | Total activity (EU) | Specific activity (EU/mg) | Yield (%) | Purification factor |
|---|---|---|---|---|---|---|---|---|---|
| Hemolysate | | 125 | 30 | 16.28 | 488.4 | 3750 | 7.68 | 100 | 1 |
| Sepharose-4B- thyrosine-sulfanilamide affinity column chromatography | hCA I | 258 | 8 | 0.34 | 2.04 | 2064 | 758.82 | 55.04 | 98.80 |
| | hCA II | 600 | 4 | 0.14 | 0.56 | 2400 | 4285.71 | 64.00 | 558.04 |

EU, enzyme unit.

Table 2. Human carbonic anhydrase isonzymes (hCA I and hCA II) inhibition data with some synthesised bromophenols, by an esterase assay with 4-nitrophenylacetate as substrate.

| | hCA-I | | hCA-II | | $K_I$ (mM) | |
|---|---|---|---|---|---|---|
| Compounds | $IC_{50}$ (μM) | $r^2$ | $IC_{50}$ (μM) | $r^2$ | hCA-I | hCA-II |
| 5 | 27.77 | 0.9925 | 142.01 | 0.8553 | 32.48 ± 13.74 | 23.03 ± 10.94 |
| 13 | 6.92 | 0.9327 | 18.52 | 0.8818 | 6.77 ± 0.87 | 3.25 ± 1.13 |
| 14 | 3.22 | 0.8986 | 24.47 | 0.9588 | 1.19 ± 1.4 | 28.56 ± 18.82 |
| 15 | 54.28 | 0.8608 | 79.42 | 0.8424 | 8.36 ± 1.21 | 42.48 ± 23.42 |
| 16 | 4.28 | 0.9411 | 63.64 | 0.9884 | 2.59 ± 1.45 | 118.41 ± 66.36 |

under *in vitro* conditions, and $IC_{50}$ values were calculated and given in Table 2.

In this study, we report the inhibitory effects of synthesised (3,4-dihydroxyphenyl)(2,3,4-trihydroxyphenyl) methanone and a series of its derivatives (**5**, **13–16**), and on the hydratase activity of hCA I and hCA II. The data in Table 2 show the following regarding the inhibition of hCA I and hCA II by bromophenol derivatives. The strongest inhibitory activity has been observed with compounds **14** and **16**, investigated in this study for the inhibition of the rapid cytosolic isozyme hCA I and hCA II (Table 2). According to two compounds, the derivative of **15** showed middle hCA I inhibitory activity with $IC_{50}$ of 54.28 μM (Table 2), whereas the remaining other derivatives were quite effective hCA I inhibitors, with $IC_{50}$'s in the range of 4.28–27.77 μM (Table 2). It is well known that phenolic compounds are effective for both hCA I and hCA II[17,18] and the other CA isoenzymes (CA I–XVI)[19,20]. In the previous reports, other phenol derivatives such as salicylic acid and paracetamol were investigated using a stopped flow, $CO_2$ hydration assay for monitoring CA inhibition[19,20]. It was reported that the presence of one or three OH moieties in phenols seemed to influence little hCA I inhibitory activities[17]. It demonstrated CA I and CA II inhibitory activities of p-hydroxybenzoic acid, p-coumaric acid, gallic, syringic acid, ferulic acid, quercetin and ellagic acid. It may be observed that small structural changes lead to impressive differences of activity. For example, ferulic acid is 2.55 times a better hCA II inhibitor compared with p-coumaric acid, of which it differs only by the presence of a supplementary methoxy moiety attached to the aromatic ring. The two isozymes showed rather different inhibition profiles with these compounds. However, hCA II was usually more easily inhibited by all the phenolic acids or polyphenols[17].

In this study, all of new synthesised (3,4-dihydroxyphenyl)(2,3,4-trihydroxyphenyl) methanone and its derivatives (**5**, **13–16**) had five –OH moieties in the both phenol rings. Bromine is electronegative rather than that of carbon atoms. The bromine group in phenolic ring pulls electrons from carbon atoms. Thus, proton abstraction ability of phenolic ring increases. This situation increases the CA isoenzyme inhibition effect of synthesised (3,4-dihydroxyphenyl)(2,3,4-trihydroxyphenyl) methanone and its derivatives compounds (**5**, **13–16**).

The synthesised (3,4-dihydroxyphenyl)(2,3,4-trihydroxyphenyl)methanone and its derivatives (**5**, **13–16**) have two phenolic rings. These synthesised phenolic compounds have been investigated as inhibitors of CA isoenzymes in this study. The rationale of investigating these compounds as CAIs lies in the fact that phenol has been shown to be the only competitive inhibitor with $CO_2$ as the substrate for the main isoform of CA such as human CA I and II[25]. In another study, Christianson and colleagues reported on the x-ray crystal structure for the adduct of hCA II with phenol, showing this compound to bind to CA by anchoring its OH moiety to the zinc-bound water/hydroxide ion of the enzyme active site through a hydrogen bond as well as to the NH amide of Thr199, an amino acid conserved in all α-CAs and critically important for the catalytic cycle of these enzymes[26]. Recently, Senturk et al. investigated the effect of some monomeric and dimeric antioxidant phenolic compounds such as 2,6-dimethylphenol, 2,6-diisopropylphenol (propofol), 2,6-di-t-butylphenol, butylated hydroxytoluene, butylated hydroxyanisole, vanillin, guaiacol, di(2,6-dimethylphenol), di(2,6-diisopropylphenol) and di(2,6-di-t-butylphenol) on hCA I and II isozymes[12]. It had been suggested that the phenolic compounds bind to enzyme active site through the phenolic hydroxyl group, which is anchored through a hydrogen bond network to the Zn(II)-bound water molecule and Thr199, the gate keeper residue of the enzyme[26,27]. In this study, the best inhibitory effect was found for di(2,6-di-t-butylphenol),

with $IC_{50}$ values of 37.5 μM (for hCA I) and 0.29 μM (for hCA II)[12].

CAIs are a class of pharmaceuticals used as antiglaucoma agents, diuretics, antiepileptics, in the management of mountain sickness, gastric and duodenal ulcers, neurological disorders or osteoporosis. Thus, the discovery of novel CAIs is of great importance for pharmacological and medicinal approaches, and many inhibitors have been designed and synthesised in the literature[12]. However, it is critically important to explore further classes of potent CAIs in order to detect compounds with a different inhibition profile when compared with sulfonamides and their bioisosteres and to find novel applications for the inhibitors of these widespread enzymes.

## Conclusions

We have synthesised (3,4-dihydroxyphenyl)(2,3,4-trihydroxyphenyl)methanone and a series of its derivatives (**5, 13–16**) and evaluated the ability of these compounds to inhibit hCA I and hCA II. All these molecules (**5, 13–16**) inhibited both the isoenzymes. The best inhibitors of hCA I were compounds **14** and **16**, which exhibited $IC_{50}$ values of 3.22 and 4.28 μM, respectively. On the other hand, the best inhibitor of hCA II was compound **13**, with an $IC_{50}$ value of 13.52 μM. These findings indicate that the synthesised phenolic compounds may be used as potent inhibitors of CA isoenzymes possibly targeting other isoforms, which have not been assayed yet for their interactions with such agents.

## Experimental section

### General information

All chemicals and solvents are commercially available and were used after distillation or treatment with drying agents. The $^1$H- and $^{13}$C-NMR spectra were recorded on a 200 (50) and 400 (100)-MHz Varian spectrometer; δ in ppm and $Me_4Si$ as the internal standard. All column chromatography was performed on silica gel (60-mesh, Merck). Preparative thick-layer chromatography (PLC) is 1 mm of silica gel 60 PF (Merck) on glass plates.

### The synthesis of compounds (5, 13–16)

The synthesis procedure of compounds (**5, 13–16**) was reported previously[4,24].

### Purification of CA isozymes from human erythrocytes by affinity chromatography

CA isozymes were purified via a simple single-step method using Sepharose-4B-L tyrosine-sulfanilamide affinity gel chromatography, described previously[12]. The purity of the enzymes was confirmed using SDS polyacrylamide gel electrophoresis. The running and stacking gels contained 10% and 3% acrylamide, respectively, and 0.1% SDS, according to the Laemmli procedure[28] described previously[14]. A 20-mg sample was applied to the electrophoresis medium. Gels were stained for 1.5 h in 0.1% Coomassie Brilliant Blue R-250 in 50% methanol and 10% acetic acid, then destained with several changes of the same solvent without the dye.

### Hydratase activity assay

The hydratase activity of hCA I and hCA II was assayed by following the hydration of $CO_2$ according to the method described by Wilbur and Anderson[29]. The activity of $CO_2$-hydratase in enzyme units (EU) was calculated by using the equation $(t_o - t_c/t_c)$, where $t_o$ and $t_c$ are the times for pH change of the nonenzymatic and the enzymatic reactions, respectively.

### Esterase activity assay

CA activity was assayed by following the change in absorbance at 348 nm of 4-nitrophenylacetate (NPA) to 4-nitrophenylate ion over a period of 3 min at 25°C using a spectrophotometer (CHEBIOS UV–VIS) according to the method described by Verpoorte et al.[30] The enzymatic reaction contained 1.4 mL 0.05 M Tris-$SO_4$ buffer (pH 7.4), 1 mL 3 mM 4-NPA, 0.5 mL $H_2O$ and 0.1 mL enzyme solution (total volume, 3.0 mL). A reference measurement was obtained by preparing the mixture without the enzyme solution. All measurements were made in triplicate. The $K_i$ values were determined from a series of experiments using three different bromophenols concentrations and 4-NPA as the substrate at five different concentrations to construct Lineweaver–Burk curves[31].

### Protein determination

The yield of protein during the purification steps was determined spectrophotometrically at 595 nm according to the Bradford method[32], using bovine serum albumin as the standard.

## Declaration of interest

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