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SHORT COMMUNICATION

Synthesis of two phloroglucinol derivatives with cinnamyl moieties as inhibitors of the carbonic anhydrase isozymes I and II: an *in vitro* study

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Abstract

Two cinnamyl-substituted phloroglucinols, 4-p-methoxycinnamyl phloroglucinol (**9**) and 4,6-bis-p-methoxycinnamyl phloroglucinol (**10**) were synthesized. Two carbonic anhydrases, human carbonic anhydrase I and II (hCA I and II), were purified. Kinetic interactions between these isozymes with **9** and **10** were investigated. These new compounds exhibited inhibitory effects on the hCA I and II enzymes' activity *in vitro*. The combination of the inhibitory effects of both phloroglucinol and p-coumaric acid groups in a single compound was explored. However, relative to the inhibitory effects of the two groups separately, compounds **9** and **10** demonstrated comparable inhibitory effects. More effective inhibitors of CAs could be created by testing these compounds on other CA isozymes.

Keywords

Enzyme inhibition, human CAI, human CAII, phloroglucinol, synthesis

History

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Introduction

Natural products have long been used as medicinal and disease-preventing molecules. Now, in modern medicinal chemistry, natural products and their derivatives are an increasing resource for early drug discovery[1]. This has resulted in a surge in the number of studies that identify active compounds from natural plant sources and investigate their properties.

Natural phenolic compounds have a significant role in the pharmaceutical industry. Their structures include one or more hydroxyl group attached to their aromatic ring. The number of hydroxyl groups and their position on the aromatic ring are important. These compounds exhibit pharmacological antioxidant, antiproliferative, anti-inflammatory and anticancer properties[2].

Phenol (**1**) and phenolic compounds (**2**–**3**) are widely used in the health sector for creating prodrugs and drugs[3]. Phloroglucinol (**3**) is a phenolic compound with three hydroxyl groups attached to its aromatic ring (Figure 1). Due to its biological properties, phloroglucinol (**3**) is used in medicine, cosmetics, paints, and pesticides[4].

Thus far, no negative effects have been reported for phloroglucinol (**3**). On the contrary, based on its catalase activation properties, phloroglucinol (**3**) has been reported to exhibit protective activity against oxidative stress resulting from H_2O_2 in cells. They also show protective effects against cell injury caused by the oxidative stress resulting from gamma radiation[4].

p-Hydroxybenzoic acid (**4**) and its derivatives (**5**–**8**) are also molecules derived from phenol. These compounds have attracted the attention of many scientists due to their promising biological features, especially their potent antioxidant properties (Figure 1), which have been the subject of many studies. These compounds have OH and OMe groups in the ortho and para positions on their aromatic rings and have been reported to show strong antioxidant activity[3,5,6].

Carbonic anhydrases (CAs; carbonate hydrolyases, EC 4.2.1.1) are a family of metalloenzymes with 16 isoforms in mammals. Their most important function is catalyzing the reversible hydration of carbon dioxide in a two-step reaction to yield bicarbonate and protons. CAs are a well-characterized type of pH regulatory enzyme found in most tissues including erythrocytes[7,8]. Many such CA isozymes have these functions show effect with the potency to be inhibited/activated for the treatment of disease such as glaucoma, edema, obesity, osteoporosis, epilepsy and cancer[8–10].

Recent studies have investigated the interaction between CA I and II isozymes and various derivatives of phenols; benzenes with different substituents; and bisphenols with antioxidant properties and their various derivatives, and salicylic acid derivatives[11–14]. Various natural and unnatural phenolic compounds have been reported to have anticancer, anticarcinogenic, antibacterial, antimutagenic, antiviral properties and anti-inflammatory activitiy[11–15].

Methods

Chemical synthesis

Commercially available reagents and solvents were of analytical grade or were purified by standard procedures prior to use. Reactions were monitored via thin-layer chromatography (TLC).

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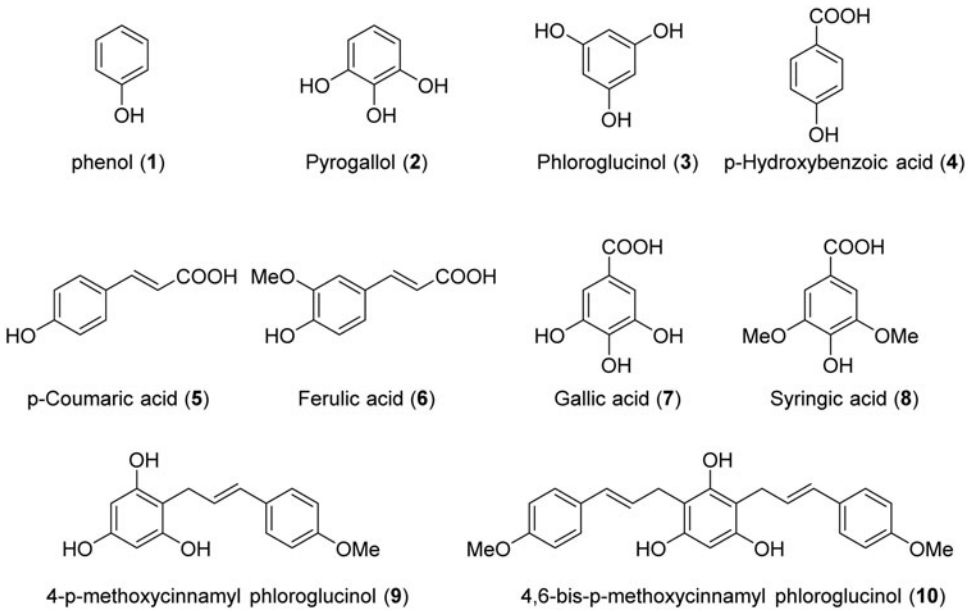


Figure 1. Chemical structures of phenolic compounds and synthesized compounds.

The ^{1}H NMR and ^{13}C NMR spectra were recorded on a 400 (100) MHz Varian spectrometer using $CDCl_3$ and Acetone-d_6. Column chromatography was performed on silica gel 60 (70–230 mesh ASTM), and TLC was carried out on silica gel (254–366 mesh ASTM). Melting points were determined on a capillary melting apparatus (Buchi 530) and are uncorrected. Infrared (IR) spectra were obtained from solutions in 0.1-mm cells with a Perkin-Elmer spectrophotometer (Waltham, MA). Elemental analyzes were performed on a Leco CHNS-932 apparatus.

*p-Methoxycinnamyl alcohol (**12**)*

To a solution of 4-methoxy-cinnamaldehyde (**11**) (5 g, 30.8 mmol) in EtOH (60 mL) at 0 °C was added sodium borohydride (1.2 g, 30.8 mmol) in one portion. The resulting mixture was stirred at rt for 30 min, then it was cooled again to 0 °C and acetone (20 mL) was added. After stirring for 10 min, sat. aqueous ammonium chloride and water were added and the mixture was extracted with EtOAc (3x60 ml). The combined organic phases were dried over Na_2SO_4 and the solvent was removed under reduced pressure to the title alcohol (4 g, 98%) as yellow solid. Rf: (0.16 20% EtOAc-Hexanes). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.33 (d, $J = 8.6$ Hz, 2H), 6.86 (d, $J = 8.6$ Hz, 2H), 6.56 (d, $J = 15.8$ Hz, 1H), 6.24 (dt, $J = 15.8$, 5.9 Hz, 1H), 4.30 (t, $J = 5.8$ Hz, 2H), 3.81 (s, 3H).

The ^{1}H NMR spectrum are in agreement with reported data by West et al.[16]

*p-Methoxycinnamyl bromide (**13**)*

To a stirred solution of **12** (483 mg, 2.94 mmol) in Et_2O (35 mL), PBr_3 (0.14 mL, 1.47 mmol) was added at 0 °C. The reaction was stirred until complete by TLC analysis (30–45 min). The mixture was quenched with a saturated NaCl (40 mL) solution and extracted with Et_2O (2 × 30 mL). The organic phase was dried with Na_2SO_4 and the solvent was evaporated under reduced pressure to yield p-methoxycinnamyl bromide (**13**) as white solid (569 mg, 85%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.33 (d, $J = 8.6$ Hz, 2H), 6.86 (d, $J = 8.6$ Hz, 2H), 6.60 (d, $J = 15.6$ Hz, 1H), 6.26 (dt, $J = 15.6$, 7.9 Hz, 1H), 4.17 (d, $J = 7.9$ Hz, 2H), 3.82 (s, 3H).

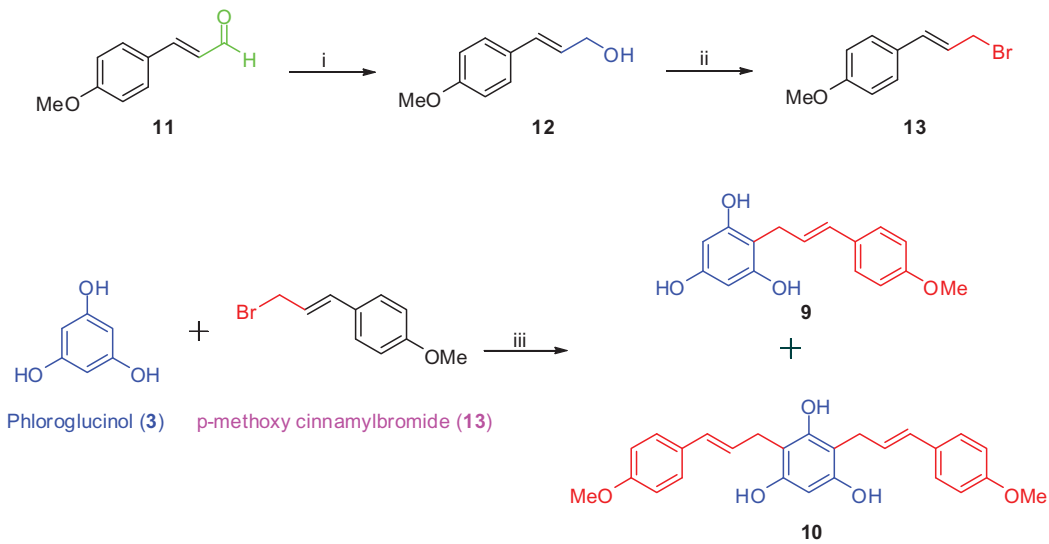
The ^{1}H NMR spectrum is in agreement with reported data by West et al.[16]

*4-p-Methoxycinnamyl phloroglucinol (**9**)*

To a suspension of NaH (35 mg, 0,87 mmol) in dry THF (4 mL), phloroglucinol (**3**) (100 mg, 0,79 mmol) was added under N_2 atm. After 5 min, a solution of **13** (179 mg, 0,79 mmol) in dry THF (4 mL) was added to the mixture. The reaction was stirred until complete by TLC analysis (17 h). The reaction was quenched NH_4Cl (10 mL) and then the mixture was neutralized with 2 M HCl until pH 1–2. The reaction mixture was extracted with EtAOc (3 × 30 mL). The organic phase was dried with Na_2SO_4 and the solvent was evaporated under reduced pressure. The crude product was purified by silica gel chromatography using EtOAc/Hexane as eluent (5%) to yield 4-p-methoxycinnamyl phloroglucinol (**9**) as a cream solid (95 mg, 16%). Melting point: 183–184 °C. Rf = 0.083 (% 60 EtOAc/Hexanes). ^{1}H NMR (400 MHz, Aseton-d_6) δ 7.97 (bs, 1H), 7.85 (bs, 1H), 7.27–7.24 (m, 2H), 6.84–6.81 (m, 2H), 6.34 (d, $J = 16$ Hz, 1H), 6.27–6.20 (m, 1H), 5.98 (s, 2H), 3.75 (s, 3H), 3.44 (d, $J = 5.2$ Hz, 2H). ^{13}C NMR (100 MHz, Aseton-d_6) δ 158.9, 156.9, 156.8, 134.5, 131.2, 128.5, 127.6, 127.0, 114.0, 94.8, 54.7, 26.2. Anal. Calculated for ($C_{16}H_{16}O_4$): C, 70.57; H, 5.92; O, 23.50; Found C, 70.52, O, 6.136.

*4,6-Bis-p-methoxycinnamyl phloroglucinol (**10**)*

To a suspension of NaH (35 mg, 0,87 mmol) in dry THF (4 mL), phloroglucinol (**3**) (100 mg, 0.79 mmol) was added under N_2 atm. After 5 min, a solution of **13** (179 mg, 0.79 mmol) in dry THF was added to the mixture. The reaction was stirred until complete by TLC analysis (17 h). The reaction was quenched NH_4Cl (10 mL) and then the mixture was neutralized with 2 M HCl until pH 1–2. The reaction mixture was extracted with EtAOc (3 × 30 mL). The organic phase was dried with Na_2SO_4 and the solvent was evaporated under reduced pressure. The crude product was purified by silica gel chromatography using EtOAc/Hexane as eluent (5%) to yield 4,6-bis-p-methoxycinnamyl phloroglucinol (**10**) as a cream solid (36 mg, 10%). Melting point: 121–122 °C. Rf = 0,2 (% 60 EtOAc/Hexanes). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.29–7.24 (m, 4H), 6.84–6.80 (m, 4H), 6.48 (d, $J = 16.0$ Hz, 2H), 6.23 – 6.15 (m, 2H), 6.02 (s, 1H), 5.36 (bs, 1H), 4.90 (bs, 1H), 3.79 (s, 6H), 3.54 (d, $J = 4.8$, 4H). ^{13}C NMR (100 MHz, $CDCl_3$) 159.3, 154.5, 153.6, 130.9, 129.8, 127.6, 125.6, 113.9, 105.2,



Scheme 1. Reagents and conditions: $NaBH_4$, EtOH, 0 °C, 30 min, 98%; (ii) PBr_3, diethylether, 0 °C, 1 h, 85%; (iii) Phloroglucinol (**3**), NaH, THF, rt, 17 h, 26%.

96.7, 55.7, 26.8. Anal. Calculated for ($C_{26}H_{26}O_5$): C, 74.62; H, 6.26; Found C, 74.66, H, 6.506.

^{1}NMR and ^{13}C NMR spectra of synthesized compounds **9** and **10** are presented in supporting information (SI).

Enzymatic inhibition studies

Purification of carbonic anhydrase isozymes from human erythrocytes by affinity chromatography

Erythrocytes suspension was obtained from the Blood Center of the Research Hospital at Erzincan University. Sepharose 4B-L-tyrosine-sulfonamide affinity chromatography was carried out as previous study[14]. The human carbonic anhydrase (hCA I and hCA II) isozymes were eluted with 1 M NaCl/25 mM Na_2HPO_4 (pH 6.3) and 0.1 M CH_3COONa/0.5 M $NaClO_4$ (pH 5.6), respectively. The absorbance of the protein in the column effluents was determined spectrophotometrically at 280 nm. All procedures were performed at 4 °C[12,17].

CA inhibition assay

Carbonic anhydrase 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.[18] 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 mL of 3 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. The inhibition effects of **9** and **10** were examined. Different inhibitor concentrations were used. Control cuvette activity in the absence of inhibitor was taken as 100%. For each inhibitor an Activity%-[Inhibitor] graph was drawn.

Protein determination

Protein during the purification steps was determined spectrophotometrically at 595 nm according to the Bradford method, using bovine serum albumin as the standard as previous study[14,19–21].

Results and discussion

The first ever synthesis of the cinnamyl-substituted phloroglucinol derivatives **9** and **10** is summarized in Scheme 1.

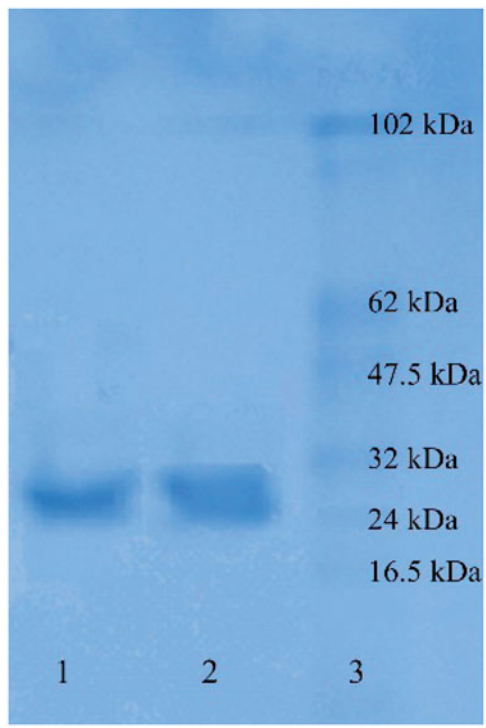


Figure 2. Polyacrylamide gel electrophoresis (PAGE) of the purified CA isozymes. Lane 1: standard proteins (62–16.5 kDa), lane 2: hCA I and lane 3: hCA II.

Both cinnamyl-substituted phloroglucinols were obtained by stirring phloroglucinol (**3**) with p-methoxycinnamyl bromide (**13**) in the presence of NaH. The p-methoxycinnamyl bromide (**13**) was prepared in two steps from p-methoxycinnamaldehyde (**11**). The ^{1}H-NMR spectrum of **9** displayed a doublet of doublets (dd) at δ 3.44 for methylene protons and a singlet for the two aromatic protons in the phloroglucinol ring (δ 5.98). The ^{1}H-NMR spectrum of **10** displayed a dd at δ 3.54 for methylene protons and a singlet for the aromatic proton in the phloroglucinol ring (δ 6.02). In this study, we compared the effects of two new synthesized compounds with the effects of phenolic compounds from previous studies on the hCA I and II isozymes. For this purpose, we extracted and purified hCA I and II from human

Table 1. Summary of the purification procedure for hCA I and hCA II.

Sample type	Total volume (ml)	Activity (EU/ml)	Protein (mg/ml)	Specific activity (EU/mg)	% Yield	Purification factor
Haemolysate	48.00	169.00	19.67	8.59	100.00	1.00
CA-I	9.60	498.00	0.57	873.70	58.90	101.70
CA-II	4.80	891.60	0.12	7430.00	52.7	864.95

Table 2. K_i values for the hCA I and hCA II inhibition data for compounds **1**, **2**, **4–10** and **AZA** by an esterase assay.

	K_i (μM)	
Compounds	hCA I	hCA II
Phenol (**1**)	10.2*	5.5*
Pyrgallol (**2**)	7.41†	0.54†
p-Hydroxybenzoic (**4**)	1061‡	675‡
p-coumaric acid (**5**)	441‡	537‡
Ferulic acid (**6**)	408‡	210‡
Gallic acid (**7**)	1052‡	758‡
Syringic acid (**8**)	919‡	695‡
4-p-Methoxycinnamyl phloroglucinol (**9**)	77.00	88.92
4,6-bis-p-methoxycinnamyl phloroglucinol (**10**)	n.d	83.22
Acetazolamide (**AZA**)	36§	3.7§

Mean from at least three determinations. Errors in the range of 3–5% of the reported value (data not shown).
*From Ref.[22–25]
†From Ref.[3]
‡From Ref.[5]
§From Ref.[15]

erythrocytes (Figure 2) and successfully synthesized compounds **9** and **10**, which contained both phloroglucinol and cinnamyl groups. Then, the effects of the synthesized compounds were determined using the esterase activity method, with 4-nitrophenyl acetate as the substrate from a previous study[14]. The purification of hCA I was achieved with a 101.7-fold purification, a specific activity of 873.70 EU mg/mL, and an overall yield of 58.90%; hCA II was purified with a 864.95-fold purification, a specific activity of 7430.00 EU mg/mL, and an overall yield of 52.70% (Table 1). The inhibitory effects of **9** and **10** on enzyme activity were tested under *in vitro* conditions. Lineweaver–Burk graphs were drawn, from which the K_i values were calculated. These results are given in Table 2.

In a previous study, the inhibition effect of compounds **1**, **2**, and acetazolamide (**AZA**) on the rapid cytosolic isozyme, hCA I, was investigated. These compounds showed a good inhibition activity (K_i of 10.2, 7.41, and 36 μM, respectively) (Table 1)[3,22–25]. In this study, the inhibition effect of the synthesized compound **9** was less than that of the compounds **1** and **2**. In another study, the inhibition effects of the compounds **4–8** on the slow cytosolic isozyme, hCA I, were found to be moderate to weak, with K_i values in the range of 408–1061 μM[5]. The presence of a –COOH group and either one or three –OH groups in the structure of these compounds changes their inhibition effects. The inhibition effects of the synthesized compound **9** were better than those of the compounds **4–8**, with K_i of 77.00 μM. K_i value for compound **10** couldn't be determined under the test conditions.

For the slow cytosolic isozyme, hCA II, the compounds **4**, **5**, **7**, and **8** were found to have average inhibition effects, with K_i values in the range of 537–758 μM. Compound **6** showed the best inhibitory activity (K_i of 210 μM) out of the compounds **4–8**[5]. In this study, the activity of the synthesized compounds **9** and **10** was less than that of the compounds **1** and **2**, but higher than that of the compounds **4–8**, with K_i values of 88.92 and 83.22, respectively.

The synthesized compounds in this study were not found to be better inhibitors than AZA, a clinically used sulfonamide. However, comparing our results with studies on molecules that were similar to the synthesized compounds, we found some interesting results. The synthesized compounds **9** and **10** contain both phloroglucinol and p-coumaric acid groups, which have both been shown to have inhibition effects on different CA isozymes in previous studies[3,5]. We had thought that when combined in a single molecule, these two groups would either increase or reduce each other's effects. However, comparing our results with the results obtained in previous studies, we found the inhibition activities of our synthesized compounds to be comparable.

The pharmacological effects of the synthesized compounds **9** and **10** could be developed clinically for hCA I and II. Due to their suitability for derivatization, these compounds could be used for the design of novel inhibitors. In addition, compounds **9** and **10** could be improved to become more effective inhibitors by adding groups to their structure with stronger inhibitory effects toward the hCA I and II isozymes. The novel therapeutic applications of these enzyme inhibitors or activators would be toward designing prodrugs and drugs in the health sector.

Declaration of interest

The authors report no declarations of interest. We thank the Scientific and Technological Research Council of Turkey (TUBITAK, Project number: 114Z554) and Erzincan University (Project number: FEN-A-300614–0098) for their financial supports of this work.

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Supplementary material available online