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Original article

Synthesis and carbonic anhydrase inhibitory properties of novel bromophenols and their derivatives including natural products: Vidalol B

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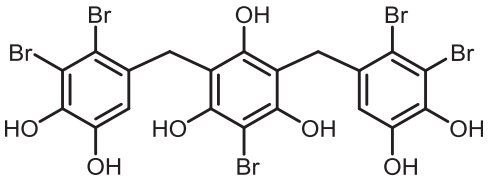
HIGHLIGHTS

- A series of bisphenol, bromophenol, and methoxyphenol derivatives were prepared.
- The first and convenient synthesis of *vidalol B* was also achieved.
- Inhibition of four human CA isozymes, with these compounds were investigated.
- The compounds were found to be promising carbonic anhydrase inhibitors.

GRAPHICAL ABSTRACT



Vidalol B

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ABSTRACT

A series of bisphenol, bromophenol, and methoxyphenol derivatives (**2**–**24**) including the natural bromophenols *vidalol B*, 3,4,6-tribromo-5-(2,5-dibromo-3,4-dihydroxybenzyl)benzene-1,2-diol (**2**) and 5,5′-methylenebis(3,4,6-tribromo-benzene-1,2-diol) (**3**) were prepared. In the current study, inhibition of four human carbonic anhydrase (hCA, EC 4.2.1.1) isozymes, I, II, IV, and VI, with these compounds **2**–**24** was investigated. The compounds **2**–**24** were found to be promising carbonic anhydrase inhibitors, some of which showed interesting inhibitory activities. Some of the compounds investigated here showed effective hCA inhibitory activity, and might be used as leads for generating novel carbonic anhydrase inhibitors which are valuable drug candidates for the treatment of glaucoma, epilepsy, gastric and duodenal ulcers, neurological disorders, and osteoporosis.

1. Introduction

Natural bromophenols, frequently isolated from red algae of the family Rhodomelaceae, have prominent biological activities [1–9]. From these natural compounds, **1** exhibits isocitrate lyase [2] and microbial [3–5] activities, while **2** and **3** show significant aldose reductase inhibitory activities (Fig. 1) [5]. Additionally, it was reported that phenolic natural [6,7,10] and synthetic dopaminergic

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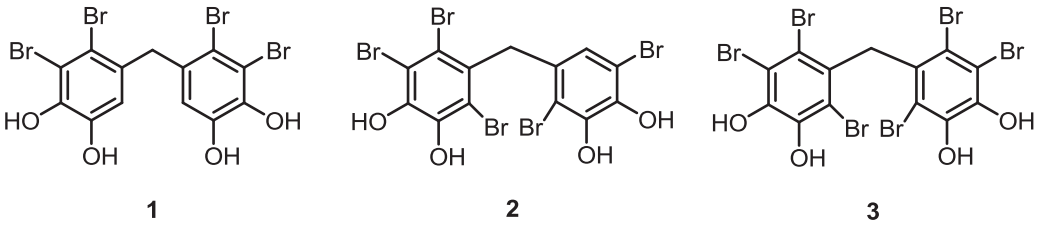


Fig. 1. Some naturally occurring bromophenols.

[11] compounds are inhibitors of human carbonic anhydrases. Antioxidant activities of **1** and **3** have also been reported [8,9]. Recently, we have achieved an alternative synthesis of **1** [8], the first total synthesis of **2** and its derivative (3,4-dibromo-5-[2-bromo-3,4-dihydroxy-6-(methoxymethyl)benzyl]benzene-1,2-diol) [12,13]. Isolation of vidalols A [**25**, 2-bromo-4-(2,3-dibromo-4,5-dihydroxybenzyl)benzene-1,3,5-triol] and B [**22**, 2-bromo-4,6-bis(2,3-dibromo-4,5-dihydroxybenzyl)benzene-1,3,5-triol], anti-inflammatory bromophenols, from the Caribbean marine red alga Vidalia, was reported previously [14].

The carbonic anhydrases (CA; carbonate hydrolase, EC 4.2.1.1) are a ubiquitous family of zinc-containing enzymes that classically participate in the maintenance of pH homeostasis in the human body, catalyzing the reversible hydration of carbon dioxide in a two-step reaction to yield bicarbonate and protons [15–17]. The enzyme plays an important role in physiological anion exchange processes [15]. At least 16 CA isozymes have been described to date in mammals, the most active ones as catalysts for carbon dioxide hydration being CA II [17–19]. CA II is found primarily in red blood cells but also in many other secretory tissues of the kidney, lung, eye, etc [15–21]. Carbonic anhydrase VI (CA VI) is a secretory enzyme that was initially described in the ovine parotid gland and, saliva and normal human serum [18]. Other CA isoforms are found in a variety of tissues where they participate in several important biological processes such as acid–base balance, respiration, carbon dioxide and ion transport, bone resorption, lipogenesis and electrolyte secretion [15–22]. Many such CA isozymes involved in these processes are important therapeutic targets with the potential to be inhibited/activated for the treatment of a range of disorders such as edema, glaucoma, obesity, cancer, epilepsy and osteoporosis [15–17,19–22].

Our group and Supuran's group recently investigated the interaction of CA I and II isozymes with several types of phenols, such as hydroxy-/methoxysubstituted benzoic acids as well as di-/tri-methoxy benzenes, natural and unnatural bromophenols, and several of its substituted derivatives, e.g., salicylates and some of their derivatives [20–22]. In the current research, the first synthesis of natural bromophenol vidalols A (**25**) and B (**22**) was studied. As CA inhibitors are valuable molecules for therapeutic and pharmacological applications, we have evaluated these bromophenol derivatives as novel carbonic anhydrase inhibitors.

We have purified human CA I, II, IV, VI (hCA I, hCA II, hCA IV and hCA VI) isoenzymes and examined the *in vitro* inhibition effects of some phenolic, bisphenol, methoxy, and bromophenol compounds mentioned above on these enzymes, using the esterase activity of hCA I, hCA II, hCA IV, and hCA VI, with 4-nitrophenylacetate as substrate.

2. Results and discussion

2.1. Chemistry

From the compounds shown in Fig. 2, **4**–**11** were synthesized by the known method [7,8,12,13] and others **12**–**17** were bought commercially.

To synthesize natural bromophenols vidalol A (**25**), B (**22**) and their derivatives, we firstly prepared (2,3-dibromo-4,5-dimethoxyphenyl)methanol (**18**) [12,13] from vanillin in four steps, and mono- and dibromo-1,3,5-trimethoxybenzene (**19** and **20**) by molecular bromination (1 and 2 eq., consecutively) of 1,3,5-trimethoxybenzene in one step. The reaction of **18** (2 equivalent) with **19** (1 equivalent) in polyphosphoric acid (PPA) by a known

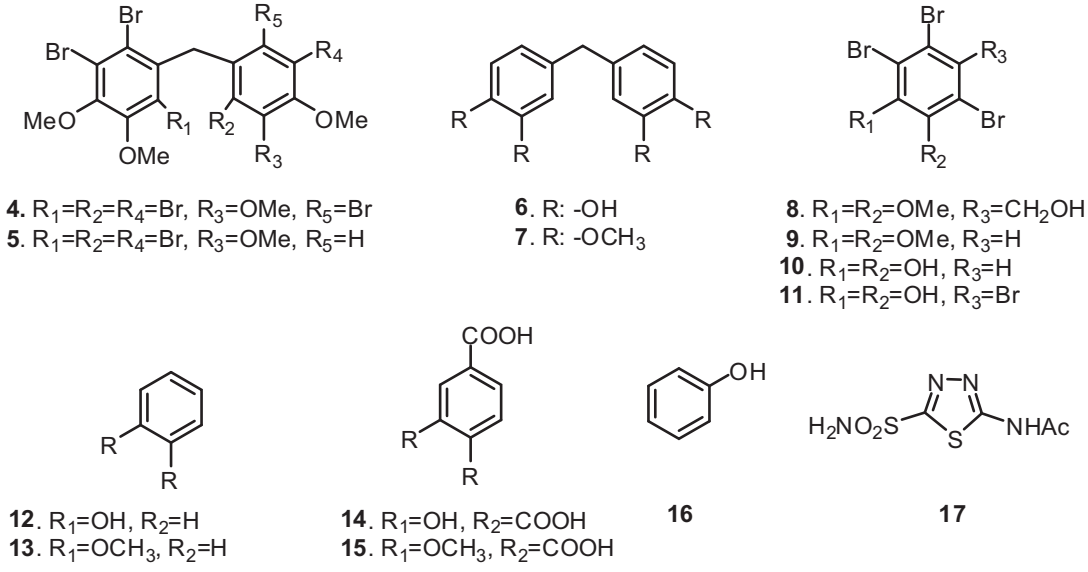


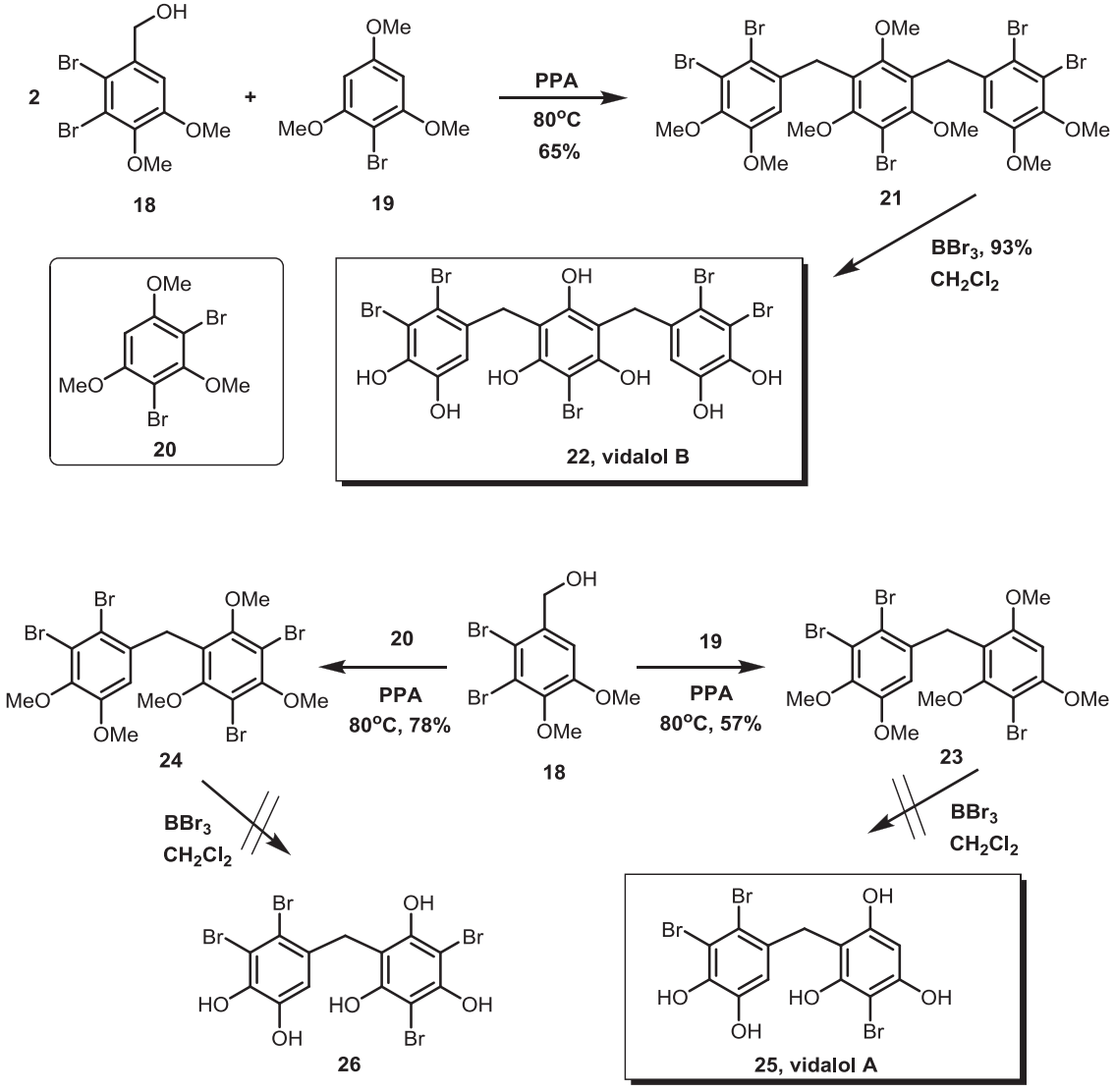
Fig. 2. From these compounds, **4**–**11** were synthesized and others **12**–**27** were bought as commercial.

method [8,12,13,23–25] gave 5,5′-(5-bromo-2,4,6-trimethoxy-1,3-phenylene)bis(methylene)bis(3,4-dibromo-1,2-dimethoxybenzene) (**21**) as a sole product. We have achieved the first convenient synthesis of the naturally occurring bromophenol vidalol B (**22**) from the reaction of **21** with BBr_3 in CH_2Cl_2 at 0–25 °C (Scheme 1). Similarly, **18** (1 equivalent) was subjected to reactions with **19** (1 equivalent) and **20** (1 equivalent), respectively, in PPA. From these reactions, 2,3-dibromo-1-(3-bromo-2,4,6-trimethoxybenzyl)-4,5-dimethoxybenzene (**23**), a precursor of vidalol A (**25**), and **24** were isolated as the sole products (Scheme 1). On the other hand, the demethylation reactions of **23** and **24** with BBr_3 in CH_2Cl_2 at 0–25 °C for the synthesis of bromophenols vidalol A (**25**) and **26** failed. From the synthesized compounds, vidalol B (**22**) is natural compound and **21** and **23** are new compounds.

2.2. CA inhibition studies

Phenol **16** binds to CA in a different manner from the classical inhibitors of the sulfonamide type, for example acetazolamide (**17**), which coordinate to the Zn(II) ion from the enzyme active site by substituting the fourth, non-protein ligand, a water molecule or hydroxide ion (Fig. 2) [7,20–22]. Supuran's group and our group have recently investigated the interactions of phenol and some of its substituted derivatives with all mammalian carbonic anhydrase enzymes [20–22], demonstrating some low micromolar/submicromolar inhibitors as well as the possibility of designing isozyme selective CAIs. The inhibition profile of various CA isozymes with this class of agents is very variable, with inhibition constants ranging from the millimolar to the submicromolar range for many simple phenols [20–22]. Thus, it seemed reasonable to us to extend the previous studies [7,8,10], including in this investigation phenolic, bisphenol, methoxy, and bromophenol compounds with clinical and antioxidant applications as food additives, such as compounds **2** and **3** and their derivatives [1–11]. Other structurally related derivatives such as **4**–**24**, were also included in our study (Fig. 2, Table 1 and Scheme 1).

The purification of the CA isozymes was performed with a simple one step method by Sepharose-4B-aniline-sulfanilamide affinity column chromatography [10,11]. hCA I was purified 110-fold with a specific activity of 941.4 EU mg^{-1} and overall yield of 62.8%. hCA II was purified 781.3-fold with a specific activity of 7165 EU mg^{-1} and overall yield of 69.4%. hCA IV was purified 86.5-fold with a specific activity of 737.2 EU mg^{-1} and overall yield of 27%. hCA VI was purified, 76.2-fold with a specific activity of



Scheme 1.

422 EU mg^{-1} and overall yield of 18.1% [10,26]. Inhibitory effects of compounds **2**, **3**, **6**, and **8**–**24** on enzyme activities were tested under *in vitro* conditions; K_I values were calculated from Lineweaver–Burk graphs [27] and are given in Table 1.

We report here the first study of the inhibitory effects of these compounds on the esterase activity of hCA I, II, IV, and VI. The sulfonamide CAI acetazolamide **AZA** [15,16] has been used as a negative control in our experiments, and for comparison reasons. The previous reports by Senturk et al. [20] investigated other antioxidant phenol derivatives (including salicylic acid and propofol) by esterase assay. The data in Table 1 show the following regarding inhibition of hCA I, II, IV and VI, with these compounds, by an esterase assay [28], with 4-nitrophenylacetate (4-NPA) as substrate:

(i) Against the slow cytosolic isozyme hCA I, compounds **7**–**9**, **12** and **21** behave as weak inhibitors, with K_I values in the range of 93.42–4003 μM [22,29,30]. Catechol **12** was an ineffective hCA I inhibitor (K_I of 4003 μM). A second group of compounds **23** and **24** showed better inhibitory activity as compared to the previously mentioned compounds, with K_I values of 57.87–61.14 μM (Table 1). Therefore, the nature of the groups in *ortho*-, *para*-, and *meta*- to the phenolic OH and OMe moiety strongly influences hCA I inhibitory activity. It is also interesting to note that the hydroxybenzoic acid derivatives **14** and **15** were much better hCA I inhibitors compared to the corresponding phenolic compounds **12** and **13** from which they were prepared. Kinetic investigations (Lineweaver–Burk plots, data not shown) indicate that, similarly to sulfonamides and inorganic anions [26,30–34], all the investigated compounds act as noncompetitive inhibitors with 4-NPA as substrate, i.e., they bind in different regions of the active site cavity as compared to the substrate. However, the binding site of 4-NPA itself is unknown, but it is presumed to be in the same region as that of CO_2, the physiological substrate of this enzyme [29–31].

(ii) A better inhibitory activity has been observed with compounds **13** and **14** for the inhibition of the rapid cytosolic isozyme hCA II (Table 1). Five derivatives, i.e., **6**, **12**, **15**, **16**, and **22**, showed moderate hCA II inhibitory activity with K_I-s in the range of 1.13–9.9 μM (Table 1). It must be stressed that K_I-s measured with the esterase method are always in the micromolar range because hCA I and II are weak esterases [30–32].

(iii) Compound **7** is a weak inhibitor of hCA IV, with a K_I value of 145.12 μM. However, again compounds **8**, **9**, and **20**–**24** are medium potency inhibitors (K_I-s of 42.26–57.61 μM), and natural bromophenol compound **22**, **AZA** show a higher affinity for this isozyme, with inhibition constant in the range of 0.578 μM (Table 1).

(iv) Phenol **16** and some of its congeners such as **7**–**9**, **12** and **13** are also weak inhibitors of the secreted isozyme hCA VI, with K_I-s of 158.592–919.182 μM. However, again the remaining derivatives **2**–**6**, **10**, **11**, **14**, **15**, and **21**–**24** are medium potency inhibitors (K_I of 2.48–45.36 μM (Table 1).

Table 1
hCA I, II, IV and VI inhibition data with the tested compounds (K_I values, μM).

Compound	hCA I	hCA II	hCA IV	hCA VI
2	34.41[a]	21.16[a]	27.48[a]	17.43[a]
3	35.12[a]	21.47[a]	28.04[a]	17.83[a]
4	43.51	35.45	32.72	29.67
5	41.36	34.62	30.78	28.17
6	18.356[b]	5.636[b]	7.64	13.692[b]
7	537.311[b]	59.107[b]	145.12	382.473[b]
8	193.24[a]	34.56	46.35	158.592[a]
9	892.109[a]	69.12	48.73	919.182[a]
10	25.341[b]	26.376[b]	13.12	11.143[b]
11	32.145[b]	29.820[b]	17.48	29.138[b]
12	4003[c]	9.9[c]	10.9[c]	606[c]
13	10.4[d]	0.50[d]	14.32	234.7
14	1.08[d]	0.47[d]	2.45	4.72
15	6.83[d]	6.18[d]	6.48	24.42
16	10.2[c]	5.5[c]	9.5[c]	208[c]
17	36.2[e]	0.37[e]	0.578[e]	0.34[e]
21	93.42	78.49	57.61	45.36
22	12.24	1.13	1.84	3.41
23	57.87	54.43	42.26	35.45
24	61.14	60.17	45.74	38.19

Mean from at least three determinations. Errors in the range of ~1% of the reported value (data not shown).
[a] Ref. [6].
[b] Ref. [29].
[c] Ref. [22].
[d] Ref. [30].
[e] Ref. [35].

The inhibition effect of halogenated sulfonamide derivatives has been investigated previously [35]. In a recent study it was reported that derivatives of salicylic acid, some phenolic compounds, and some benzoic acid derivatives [20–30], a simple compound lacking the sulfonamide, sulfamate, or related functional groups that are typically found in all known CA inhibitors, act as a CA I inhibitor, and could represent the starting point for a new class of inhibitors that may have advantages for patients with sulfonamide allergies [31]. The sulfonamide zinc-binding group is thus superior to the hydroxyl for generating CA inhibitors with a varied and sometimes isozyme-selective inhibition profile against mammalian enzymes [32–38]. However, it is critically important to explore further classes of potent CAIs in order to detect compounds with a different inhibition profile compared to the sulfonamides and their bioisosteres and to find novel applications for the inhibitors of these widespread enzymes [37,38].

3. Conclusions

The first and convenient synthesis of the naturally occurring bromophenol *vidalol B* (**22**) from the corresponding compounds was achieved. Carbonic anhydrase inhibitory properties of a series of bisphenol, bromophenol and methoxyphenol derivatives (**2**–**24**) including natural bromophenols were investigated.

Phenolic, bisphenol, methoxy, and bromophenol compounds influence the activity of hCA isozymes due to the presence of different functional groups (OH, OMe, COOH, and Br) in their aromatic scaffold. Our findings indicate thus another class of possible CAIs of interest, in addition to the well-known sulfonamides/sulfamates/sulfamides, the phenols/biphenyl diphenols bearing bulky *ortho* moieties in their molecules. Compounds **6**–**15** investigated here showed effective hCA I and II inhibitory activity [22,29–32,37], in the low micromolar range, by the esterase method, which usually gives K_I-s an order of magnitude higher compared to the CO_2 hydrase assay [31]. These findings point out that substituted phenolic, bisphenol, methoxy, and bromophenol compounds may be used as leads for generating potent CAIs eventually targeting other isoforms that have not been assayed yet for their interactions with such agents.

4. Experimental

Sepharose 4B, protein assay reagents, 4-nitrophenylacetate and chemicals for electrophoresis were purchased from Sigma–Aldrich Co. All other chemicals and solvents are commercially available and were used after distillation or treatment with drying agents. Melting points were determined on a capillary melting apparatus (BUCHI 530) and are uncorrected. IR spectra were obtained from

solutions in 0.1 mm cells with a *Perkin–Elmer* spectrophotometer. The ^1H and ^{13}C NMR spectra were recorded on a 400 (100)-MHz *Varian spectrometer*; δ in ppm, Me_4Si as the internal standard. Elemental analyses were performed on a Leco CHNS-932 apparatus. All column chromatography was performed on silica gel (60-mesh, Merck). PLC is preparative thick-layer chromatography: 1 mm of silica gel 60 PF (Merck) on glass plates.

4.1. Synthesis

The compounds **4**–**11** were synthesized by the known method [7,8,12,13].

4.1.1. Standard procedure: synthesis of 5,5′-(5-bromo-2,4,6-trimethoxy-1,3-phenylene)bis(methylene)bis(3,4-dibromo-1,2-dimethoxybenzene) (*21*)

Polyphosphoric acid (PPA), prepared from conc. H_3PO_4 (85%, 2.32 g, 24 mmol) and P_2O_5 (4.15 g, 29 mmol), was heated to 80 °C in a beaker (50 mL) [12,13,23–25]. To this mixture were added **19** (0.5 g, 2.02 mmol) and **18** (1.32 g, 4.05 mmol) consecutively and quickly. The mixture was stirred with a glass rod at 80 °C for 1 h and cooled to room temperature (RT). A mixture of water and ice (50 mL) was carefully added to the cooled mixture. The organic phase was separated and then the water phase was extracted with EtOAc (2 × 40 mL). The combined organic layers were dried over $MgSO_4$ and the solvent was evaporated. The product **21** (1.01 g, 65%) was obtained and crystallized from ethyl acetate/hexane (3/1) as pale yellow crystals. Mp: 136–137 °C, ^1H NMR (400 MHz, $CDCl_3$) δ 6.39 (s, 2H), 4.16 (s, CH_2, 4H), 3.80 (s, OCH_3, 6H), 3.76 (s, OCH_3, 6H), 3.59 (s, OCH_3, 6H), 3.57 (s, OCH_3, 3H), ^{13}C NMR (100 MHz, $CDCl_3$) δ 157.97(C), 156.53 (C), 152.36 (C), 146.20 (C), 137.24 (2C), 124.65 (C), 121.79 (C), 117.54 (C), 112.68 (CH), 61.84 (OCH_3), 61.28 (OCH_3), 60.50 (OCH_3), 56.23 (OCH_3), 32.72 (CH_2). IR (CH_2Cl_2, cm^{-1}): 2999, 2937, 2868, 2840, 2591, 2283, 2013, 1582, 1549, 1466, 1422, 1404, 1372, 1321, 1283, 1255, 1221, 1195, 1161, 1105, 1082, 1058, 1005, 966, 950, 903, 881, 843, 819, 804, 775, 737, 704, 662, 591, 510, 487. Anal. Calcd for ($C_{27}H_{27}Br_5O_7$): C 37.58, H 3.15; Found C 37.65, H 3.16.

4.1.2. Synthesis of 2-bromo-4,6-bis(2,3-dibromo-4,5-dihydroxy benzyl)benzene-1,3,5-triol (vidalol B, *22*)

A solution of **21** (0.500 g, 0.58 mmol) in CH_2Cl_2 (10 mL) was cooled to 0 °C and then a solution of BBr_3 (0.65 mL) in CH_2Cl_2 (7 mL) was added dropwise under N_2 (g) over 5 min. After the cold bath was removed, the mixture was stirred at RT and under N_2 for 1 day. Methanol (15 mL) was slowly added over 15 min at 0 °C and then the solvent was evaporated. After water (40 mL) and EtOAc (50 mL) were added, the mixture was shaken. The organic phase was separated and the water phase was extracted with EtOAc (2 × 30 mL). The combined organic phases were dried over $MgSO_4$ and the solvent was evaporated. Natural product **22** (0.412 g, 93%) was obtained as brownish solid. Mp: 188.5–189 °C, ^1H NMR (400 MHz, CD_3COCD_3) δ 8.59 (s, 2OH), 8.07 (s, 2OH), 7.83 (s, OH), 7.77 (s, 2OH), 6.38 (s, 2H), 4.04 (s, CH_2, 4H), ^{13}C NMR (100 MHz, $CDCl_3$) δ 154.76 (C), 151.88 (C), 144.70 (C), 142.59 (C), 132.46 (2C), 115.96 (C), 114.20 (CH), 112.93 (C), 106.67 (C), 31.68 (CH_2), IR (CH_2Cl_2, cm^{-1}): 2975, 2863, 2112, 1646, 1468, 1401, 1340, 1273, 1220, 1172, 1105, 1055, 1033, 1014, 915, 856, 666, 551, 434, 408, 400.

4.1.3. Synthesis of 2,3-dibromo-1-(3-bromo-2,4,6-trimethoxy benzyl)-4,5-dimethoxybenzene (*23*)

The standard procedure described above (Section 4.1.1.) for **21** was applied, but the ratio of reactants **18** and **19** was 1. Compound **23** (57%) was crystallized from ethyl acetate/hexane (3/1) as colorless crystals. mp: 123–125 °C, ^1H NMR (400 MHz, $CDCl_3$) δ 6.38 (s, 1H), 6.33 (s, 1H), 4.06 (s, CH_2, 2H), 3.94 (s, OCH_3, 3H), 3.80 (s, OCH_3, 3H), 3.79 (s, OCH_3, 3H), 3.71 (s, OCH_3, 3H), 3.62 (s, OCH_3, 3H), ^{13}C NMR (100 MHz, $CDCl_3$) δ 158.43(C), 157.31 (C), 156.47 (C), 152.27 (C), 145.74 (C), 137.80 (C), 121.47 (C), 117.45 (C), 114.98 (C), 112.02 (CH), 98.21 (C), 9.59 (CH), 61.17 (OCH_3), 60.46 (OCH_3), 56.47 (OCH_3), 56.08 (OCH_3), 55.94 (OCH_3), 31.84 (CH_2), IR (CH_2Cl_2, cm^{-1}): 3000, 2937, 2840, 2593, 2427, 2284, 2120, 2017, 1963, 1913, 1592, 1549, 1463, 1435, 1422, 1394, 1372, 1337, 1321, 1282, 1254, 1199, 1164, 1107, 1057, 1008, 963, 946, 895, 857, 802, 757, 739, 656, 588, 491, 458, Anal. Calcd for ($C_{18}H_{19}Br_3O_5$): C 38.95, H 3.45; Found C 38.90, H 3.46.

4.1.4. Synthesis of 1,3-dibromo-5-(2,3-dibromo-4,5-dimethoxy benzyl)-2,4,6-trimethoxybenzene (*24*)

The standard procedure described above (Section 4.1.1.) for **21** was applied, but, the ratio of reactants **18** and **20** was also 1. The known [39] compound **24** (78%) was crystallized from ethyl acetate/hexane (3/1) as colorless crystals. mp. 122–124 °C, ^1H NMR (400 MHz, $CDCl_3$) δ 6.36 (s, 1H), 4.17 (s, CH_2, 2H), 3.93 (s, OCH_3, 3H), 3.80 (s, OCH_3, 3H), 3.73 (s, OCH_3, 6H), 3.65 (s, OCH_3, 3H), ^{13}C NMR (100 MHz, $CDCl_3$) δ 156.55(C), 154.85 (C), 152.43 (C), 146.17 (C), 136.81 (C), 125.66 (C), 121.77 (C), 117.40 (C), 112.35 (CH), 109.62 (C), 61.27 (2 OCH_3), 60.75 (OCH_3), 60.50 (OCH_3), 56.21 (OCH_3), 33.00 (CH_2), IR (CH_2Cl_2, cm^{-1}): 3000, 2965, 2936, 2853, 2581, 2025, 1582, 1550, 156, 1422, 1397, 1382, 1322, 1284, 1264, 1198, 1164, 1085, 1058, 1003, 970, 947, 904, 868, 841, 810, 767, 713, 686, 672, 593, 497. Anal. Calcd for ($C_{18}H_{18}Br_4O_5$): C 34.10, H 2.86; Found C 34.15, H 2.85.

4.2. Purification of carbonic anhydrase isoenzymes from human by affinity chromatography

Purification of hCA I and hCA II was previously described [10]. Fresh citrated human whole blood was obtained from the Blood Center of the Research Hospital at Atatürk University. Cells were washed three times by centrifugation at 1000 × *g* at 4 ± 6 °C, for 20 min in four volumes of 25 mM Na_2HPO_4 (pH = 7.4) buffer. The supernatant and fluffy coat were removed. The erythrocytes were lysed in 10 volumes of 5 mM Na_2HPO_4 (pH = 7.4) buffer, containing 1 mM EDTA. After 20 min the hemolysate was centrifuged at 10.000 × *g* for 60 min. The particulate fraction was washed four times in the same buffer. The membranes were centrifuged down at 15.000 × g for 60 min. pH was adjusted to 8.3 with solid Tris. A sepharose-4B-aniline-sulfanilamide affinity column was equilibrated with 25 mM Tris–HCl/0.1 M Na_2SO_4 (pH 8.3). The affinity gel was washed with 25 mM Tris–HCl/25 mM Na_2PO_4 (pH 8.3). Finally, human carbonic anhydrase IV (hCA IV) isozyme was eluted with 25 mM Tris–HCl/0.5 M $NaClO_4$ (pH 7.4). Fresh non-citrated human whole blood was obtained from the Blood Center of the Research Hospital at Atatürk University. The blood samples were centrifuged at 5000 rpm for 15 min and the precipitant was removed. The serum was isolated. The pH was adjusted to 8.7 with solid Tris. A sepharose-4B-aniline-sulfanilamide affinity column was equilibrated with 25 mM Tris–HCl/0.1 M Na_2SO_4 (pH 8.7). The affinity gel was washed with 25 mM Tris–HCl/22 mM Na_2SO_4 (pH 8.7). The human carbonic anhydrase (hCA VI) isozyme was eluted with 0.25 M H_2NSO_3H/25 mM Na_2HPO_4 (pH = 6.7) [18].

4.3. CA inhibition

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 according to the method described by Verpoorte et al. [28]. 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 of H_2O and 0.1 mL of enzyme solution.

A reference measurement was obtained by preparing the same cuvette without enzyme solution. The inhibitory effects of compounds **2**–**24** were examined. All compounds were tested in triplicate at each concentration used. 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. The curve-fitting algorithm allowed us to obtain the IC_{50} values, working at the lowest concentration of substrate of 0.15 mM, from which K_I values were calculated by using the Cheng–Prusoff equation [36]. The catalytic activity (in the absence of inhibitors) of these enzymes was calculated from Lineweaver–Burk plots, as reported earlier, and represent the mean from at least three different determinations. The enzymes used here were purified from human blood as described earlier.

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Appendix A. Supplementary data

Supplementary data related to this article can be found online at doi:10.1016/j.ejmech.2012.05.025.

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