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Full Paper

Synthesis and Biological Evaluation of Novel Bischalcone Derivatives as Carbonic Anhydrase Inhibitors

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Design and synthesis of a new type of bischalcones as an alternative to natural and synthetic bischalcones are reported for the first time. Key steps involved the solvent-free Claisen–Schmidt condensation of chalcones, and the successful first application of the diazotization–diazocoupling reaction in the synthesis of C–N=N–C-linked bischalcones by simple structural modification of *p*-aminoacetophenone. The structures of all compounds were confirmed by means of FT-IR, ^{1}H and ^{13}C NMR, ESI/MS, and elemental analysis. In addition, the newly synthesized compounds were screened for carbonic anhydrase inhibition activities. Almost all bischalcones exhibited moderate-to-good inhibitory activities.

Keywords: Bischalcone / Carbonic anhydrase inhibition / Diazotization / Solvent-free

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Introduction

Chalcone and its analogs are of considerable interest for both synthetic organic and medicinal chemists due to their remarkable range of biological and pharmaceutical properties. Chalcone derivatives have been used to treat certain kinds of cancer [1], diabetes [2], and to prevent cardiovascular diseases [3]. Some of its derivatives show antimicrobial [4], antibacterial [5], antifungal [6], and anti-HIV activity [7]. Bischalcones are well represented in the Anacardiacea family. The *Rhus* genus is also a rich source for biflavonoids and bischalcones. In general, naturally occurring bischalcones, rhuschalcone **(I)**, littorachalcone **(II)**, and verbenachalcone **(III)** as depicted in Figure 1 carry either a C–O–C or C–C linkage between the two chalcone fragments [8–11]. Rhuschalcone II–VI has been isolated from the root bark of *Rhus* pyroides and synthetic analogs have been shown to possess strong antiplasmodial and moderate antiproliferative, antiprotozoal activities. In addition, rhuschalcone **(I)** displays important cytotoxic activity against the HT29 and HCT-116 colon tumor cell lines [9, 11, 12]. Its dihydro analogs, bearing diaryl ether linkage, both littorachalcone **(II)** and verbenachalcone **(III)**, showed a significant extension of nerve growth factor-mediated neurite outgrowth from the PC12D cells [10, 11]. They were synthesized by phenolic oxidation of the dihalogenated phenols. Recently, a number of pharmacologically interesting bischalcone analogs were synthesized with structural modifications to improve their anti-cancer potential and chemoprotective properties [13]. Examples of these transformations are formed by joining two chalcone moieties together with different linkages such as bi-aryl, di-aryl ether, methylene, the carbon chains of varying lengths, Mannich base, etc. using Suzuki–Miyaura [9], Mannich [8], Ullmann [11], and Williamson [14] reactions. Synthetic bischalcone bearing piperazine Mannich base linkage, which is (*E*)-1-(4-(4-(5-cinnamoyl-2-hydroxybenzyl)-piperazin-1-yl)phenyl)-3-phenylprop-2-en-1-one **(IV)**, showed inhibition of nitric oxide (NO) production in microglial cells and for *in vitro* anticancer activity. 1,2-Di(2′-benzoyl-4-styryloxy)-ethane and its derivatives **(V)** with a structure of two chalcone moieties connected by the carbon chains of varying lengths have

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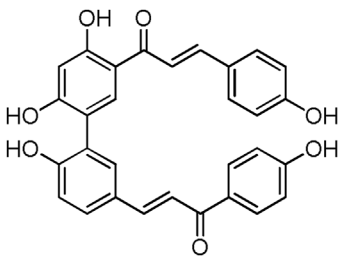
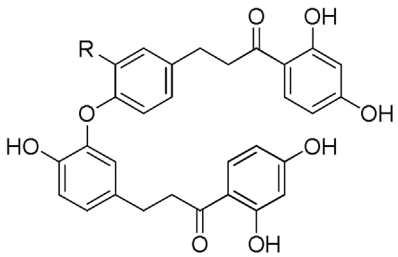
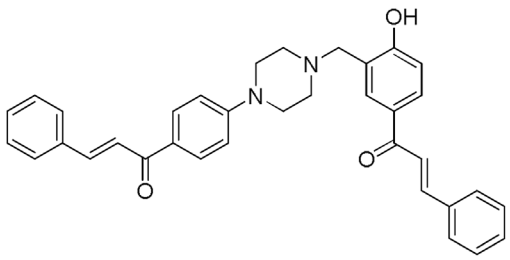


I Rhuschalcone VI

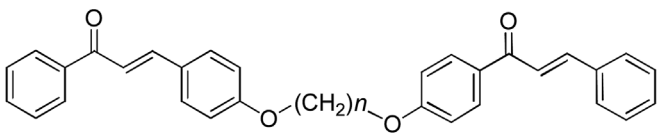


II Littorachalcone, R = –H

III Verbenachalcone, R = –OMe



IV



n = 2, 3, 4, 5, 6

V

Figure 1. Natural and synthetic bischalcones.

been synthesized through the Williamson reaction and they have been shown to possess antimicrobial activity [14] (Fig. 1).

In addition, aromatic and heteroaromatic azo compounds constitute the most important class of synthetic dyes, which are used commonly in applications such as organic dyes [15, 16], indicators [17], and therapies [18]. They are also utilized in the area of nonlinear optics [19] and optical storage media [20]. Moreover, azo compounds are also involved in a number of biological reactions such as inhibition of DNA, RNA and protein synthesis, carcinogenesis, and nitrogen fixation [21–23]. For this reason, the synthesis of aromatic azo compounds has attracted great attention.

Carbonic anhydrase (carbonate hydrolase, EC 4.2.1.1; CA) is a zinc(II)-dependent enzyme that catalyzes the reversible hydration of carbon dioxide to hydrogencarbonate and a proton [24]. CA isozymes have been the targets of drug developments for the treatments of glaucoma, epilepsy, high-altitude sickness, as well as cancer [24, 25]. CA isozymes are involved in the pH homeostasis, ion transport, water and electrolyte balance, bone resorption, calcification, and tumorigenesis [24]. In a recent preliminary work from our group, we investigated the interaction between natural phenolic compounds, amino acid sulfonamide derivatives, hydroxy/methoxy organic compounds, and some nitroaromatic compounds with two cytosolic catalytically active isoforms (CA I and II) of the metalloenzyme CA [26–33].

Over the last few years, bischalcone and its derivatives have gained much interest because of their wide range of applications in synthetic and pharmaceutical chemistry. Current research focuses on the transformations of chalcones into bischalcones in particular. Thus, we utilized the diazotization and diazocoupling reaction for the synthesis of bischalcones. Moreover, from a synthetic aspect, this effort provided an excellent opportunity to achieve the new types of diazotization

and diazocoupling reaction in the synthesis of novel type bischalcones, which have C–N=N–C linked with various substitutions in the B-ring and D-ring of the chalcone moiety.

Results and discussion

Chemistry

The convergence of the strategy enables the synthesis of a new type of bischalcones bearing a C–N=N–C linkage with simple structural modification of *p*-aminoacetophenone. In the synthetic route, the first step involves its diazotization and azo coupling. Bisketone (**3**) has been converted into the corresponding diazonium salt by using a treatment with sodium nitrite and HCl at 0–5°C to give the corresponding benzenediazonium chlorides, which were used directly in the next step without any further purification [34]. Subsequently, the diazonium salt was coupled with 3-hydroxyacetophenone in a dilute NaOH solution at 0–5°C for 4 h leading to the formation of bisketone (**3**). The crude product was purified by recrystallization. In the previous studies, bisketone (**3**) was first found in low yield as a rearrangement product of 4,4′-diacetylazoxybenzene with sulfuric acid [35]. However, there are no earlier reports regarding its synthesis. Step two, the transformation of bisketone into bischalcone includes the Claisen–Schmidt reaction of two equivalents of the corresponding benzaldehydes with bisketone using the grindstone technique. This technique is superior to the conventional method as it is eco-friendly, high yielding, non-hazardous, simple, and convenient [9, 36, 37]. Therefore, the grindstone technique was used for the synthesis of the new type bischalcones. Claisen–Schmidt condensation, which is the most important reaction in the formation of 1,3-diphenyl-2-propene-1-ones (chalcones), aldehydes-bearing methoxy, methyl, and halogens group, reacted with bisketone (**3**) in the presence of the solid KOH in combination with grinding under solvent-free conditions at room temperature. Thus, the synthesis of the unsymmetrical bischalcone, including the C–N=N–C bond, was achieved in step two (Scheme 1). The structures of all the compounds were confirmed by FT-IR, ^{1}H and ^{13}C NMR, ESI/MS, and elemental analysis.

The synthetic route to the bischalcones is outlined in Table 1. The crude product was contaminated with some starting materials, which could easily be removed by recrystallization two times with EtOH/H_2O to obtain red crystals. The ESI/MS spectra of the compounds showed $[M+H]^+$ peaks corresponding to their molecular formula. The NMR spectra (^{1}H and ^{13}C NMR) are consistent with the proposed structures. The *trans*-(*E*) geometry of the bischalcones **4–14** double bond was evident by the large olefinic coupling constant between the relevant signals in the ^{1}H NMR spectrum ($J = 15.0$–16.4 Hz).

Biological evaluation of the synthesized and reference compounds for CA inhibitory activity

In the current study, we report the synthesis of bischalcone derivatives containing a methoxy group (**3–14**). We determined the inhibitory effects of these compounds on two α-CA isozymes, hCA I and hCA II.

It is known that CA has been purified many times from different organisms and the effects of various chemicals, pesticides, and drugs have been investigated on its activity [26–33]. In this study, CA I and II were purified from human erythrocytes [27], and the activity of the effluents was determined by the hydratase method, with CO_2 as substrate and further kinetic studies were performed using the esterase activity method, with 4-nitrophenyl acetate (NPA) as substrate [21]. Here, we report the inhibitory effects of bischalcone derivatives on the CA esterase activity of isoforms hCA I and II. Data of Table 1 show the following regarding inhibition of hCA I and II with compounds **3–14**:

(1) Against the slow cytosolic isozyme hCA I, compounds **5**, **7**, and **11** behaved as weak inhibitors. A second group of derivatives, including **3**, **6**, **9**, **10**, **13**, and acetazolamide (AZA), showed better inhibitory activity compared to the previously mentioned compounds, with K_Is in the range of 165.35–274.19 nM. Molecules **4**, **8**, **12**, and **14** were among the best inhibitors in this series of nitrobenzene compounds. Data of Table 1 also show that similarly to AZA, some of the investigated bischalcone compounds act as competitive inhibitors with 4-NPA as substrate, that is, they bind to the same regions of the active site cavity as 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 [38]. Similarly to methoxyphenolic compounds investigated earlier by us [39–41], these compounds act as competitive inhibitors with 4-NPA as substrate, that is, they bind to same regions of the active site cavity as compared to the substrate (Table 1).

(2) A rather similar activity of these compounds has been observed also for the inhibition of the rapid cytosolic isozyme, hCA II (Table 1). Thus, a first group of derivatives, **3**, **5**, **7**, **9**, and **14**, showed modest hCA II inhibitory activity with K_Is in the range of 307.52–645.39 nM (Table 1), whereas the remaining seven derivatives, the same compounds acting as efficient hCA II inhibitors, showed K_Is in the range of 154.45–268.87 nM. Structure–activity relationship was thus quite similar in these small groups of methoxy derivatives, for both the inhibition of hCA I and II, although differences of affinity between the two isozymes are evident. Again most of these compounds acted as competitive inhibitors with 4-NPA as substrate (Table 1).

Conclusion

In conclusion, an environmentally benign approach, and synthesis of new type bischalcones were successfully accomplished via diazotization and diazocoupling by using the grinding method. This synthesis constitutes the first

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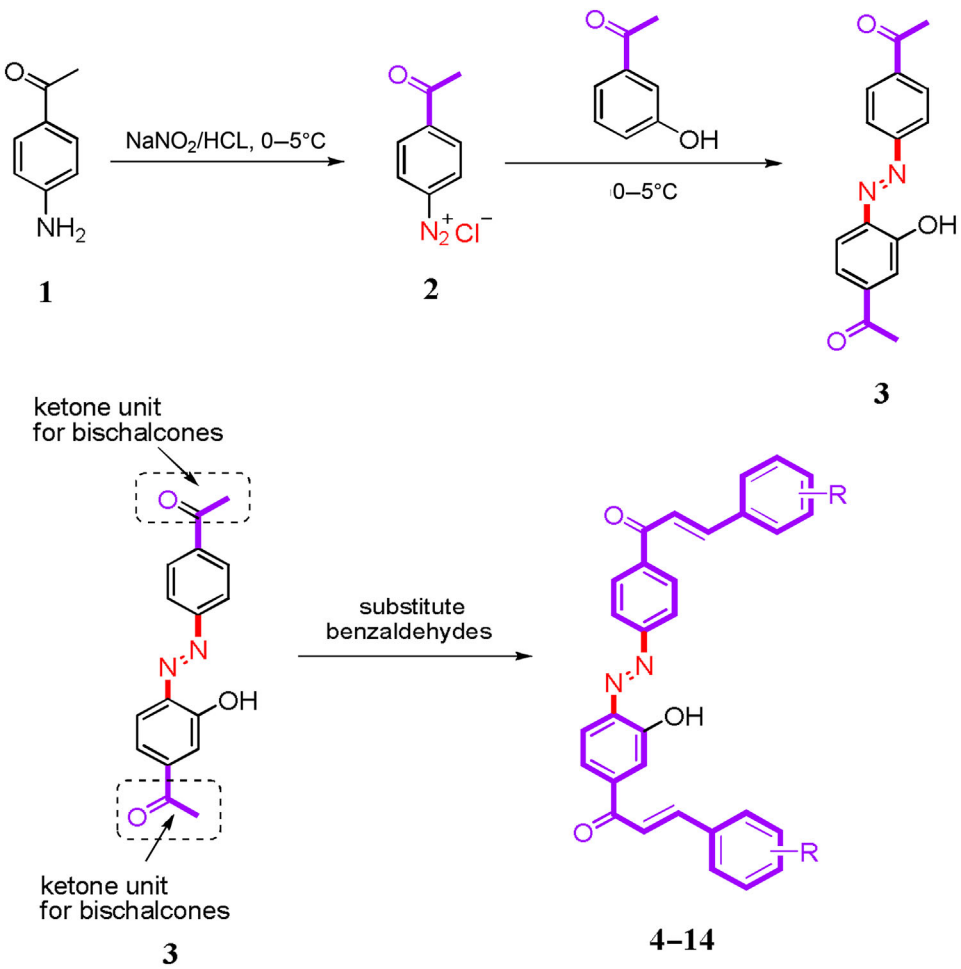

Scheme 1. Synthesis of new type bischalcone derivatives using the diazotization–diazocoupling reaction.

application of the diazotization–diazocoupling reaction in the synthesis of C–N=N–C-linked bischalcones. The newly synthesized compounds were also evaluated for CA inhibition activity. Bischalcones are the most valuable synthons for synthetic organic chemists, and by means of proper planning and design of these active compounds can give inspiration for the synthesis of many important and exotic heterocyclic compounds as pharmaceuticals.

Experimental

Chemistry

^{1}H and ^{13}C spectra were recorded on a Varian Mercury 200 (50)-MHz and Bruker Ascend 400 (100)-MHz spectrometers and chemical shifts were reported (λ) relative to Me_4Si as internal standard. High-resolution mass spectrometric analysis was carried out on an Agilent 1260 Infinity Series Q-TOF LC/MS (ESI/MS). The elemental analyses were performed on a Costech ESC 4010 instrument. The IR spectra were determined using a Perkin Elmer 1600 Fourier Transform-infrared (FT-IR) spectrophotometer on a KBr disc. Melting points were determined by using a Barnstead electrothermal 9200 series digital apparatus. Ultraviolet–visible (UV–Vis) absorption spectra were recorded on a Unicam UV2-100 spectrophotometer. CA inhibitory activity was determined according to Verpoorte et al. [38].

The InChI codes of the investigated compounds are provided as Supporting Information.

*Synthesis of bisketone (**3**)*

4-Aminoacetophenone (2 g, 14.8 mmol) was dissolved in 5 mL of concentrated HCl. The solution was cooled down in a ice-salt bath and then cold solution of $NaNO_2$ (1 g, 14.8 mmol) in 10 mL of water was slowly added. The reaction mixture resulted was stirred for 3 h at 0–5°C. The resulting diazonium salt was cooled in ice-salt bath. 3-Hydroxyacetophenone (2 g, 14.8 mmol) was dissolved in a dilute NaOH solution and

Table 1. Characterization and K_I values of the tested compounds.

Product	R	Yield (%)	M.p. (°C)	UV[a] λ_{nm} (log ε)			hCA I K_I (nM)[b]	hCA II K_I (nM)[b]
3	–	62	208–210	537 (3.4)	–	–	274.19	645.39
4	H	60	106–108	563 (3.4)	–	–	112.34	154.45
5	2-OMe	77	92–94	358 (4.6)	566 (3.4)	–	325.12	418.06
6	3-OMe	88	87–89	290 (4.3)	375 (4.4)	571 (3.5)	165.35	227.56
7	4-OMe	84	142–144	354 (4.6)	605 (3.5)	–	308.88	307.52
8	2,3-OMe	83	80–82	312 (4.5)	378 (4.6)	608 (3.7)	111.22	193.19
9	2,4-OMe	79	102–104	379 (4.6)	554 (3.5)	–	177.91	446.62
10	2,5-OMe	61	88–90	379 (4.6)	570 (3.2)	–	188.56	208.53
11	3,4-OMe	86	97–99	371 (4.6)	567 (3.6)	–	323.30	182.31
12	3,5-OMe	80	136–138	326 (4.4)	379 (4.4)	–	72.90	166.54
13	2,3,4-OMe	72	90–92	378 (4.6)	377 (4.6)	566 (3.4)	179.11	268.87
14	3,4,5-OMe	65	145–147	375 (4.6)	554 (3.5)	–	103.55	319.13
AZA	–	–	–	–	–	–	250.00	12.00

[a] The UV–Vis absorption spectra were recorded using DMF in concentration 1.10^{-8} M.
[b] Mean from at least three determinations. Errors in the range of ~3% of the reported value (data not shown).

cooled in ice-salt bath and then the cold diazonium solution was added to this cooled solution dropwise at 0–5°C. After stirring the reaction mixture for 4 h, pH of the reaction mixture was adjusted at 4–5 by the simultaneous addition of a saturated sodium acetate solution. Subsequently, it was stirred for 2 h. The precipitate was filtered, dried, and recrystallized from EtOH/H_2O (1:1).

*General methods for the synthesis of bischalcones **4–14***

Bisketone (**3**) (2 mmol), substituted benzaldehyde (4 mmol), and potassium hydroxyde (4 mmol) are ground in a mortar with a pestle at room temperature for 10–15 min to obtain a homogeneous mixture. The progress of reaction was monitored by TLC. After completion of the reaction, the mixture was poured into ice water (10 mL), adjusted to pH 2–3 with 1 M HCl. The dark red solid thus obtained was filtered, washed with several times water, dried, and crystallized from EtOH/H_2O (1:1).

*(E)-1-(4-((4-Acetyl-2-hydroxyphenyl)diazenyl)phenyl)-ethanone (**3**)*

Dark red solid, FT-IR (KBr, cm^{-1}): 3356, 1681, 1678, 1268. ^{1}H NMR (200 MHz, $CDCl_3$): δ 8.10 (d, J = 8.2, 2H), 7.83 (d, J = 8.2, 2H), 7.76 (d, J = 9.0, 1H), 7.00 (d, J = 9.0, 1H), 6.90 (s, 1H), 2.60 (s, 3H), 2.51 (s, 3H). ^{13}C NMR (50 MHz, $CDCl_3$): δ 202.85, 198.07, 162.06, 154.83, 142.67, 142.39, 138.58, 130.34, 123.14, 121.91, 118.71, 114.42, 32.92, 27.64. ESI/MS (*m/z*): $[M+H]^+$ 283.08. Anal. calcd. for $C_{16}H_{14}N_2O_3$: C, 68.07; H, 5.00; N, 9.92. Found: C, 68.10; H, 5.01; N, 9.96.

*(E)-1-(4-((E)-(4-Cinnamoyl-2-hydroxyphenyl)diazenyl)-phenyl)-3-phenylprop-2-en-1-one (**4**)*

Dark red solid, FT-IR (KBr, cm^{-1}): 3400, 3056, 1676, 1533. ^{1}H NMR (200 MHz, DMSO-d_6): δ 8.21 (d, J = 8.6, 1H, ArH), 7.92 (d, J = 8.6, 1H, ArH), 7.85–7.87 (m, 7H, ArH), 7.69 (m, 2H, ArH), 7.50 (B part of AB system, J = 16.0, 1H), 7.46 (m, 5H, ArH), 7.29 (A part of AB system, J = 16.0, 1H), 7.07 (s, 1H, ArH). ^{13}C NMR (50 MHz, DMSO-d_6): δ 195.24, 189.35, 161.16, 154.36, 144.81, 143.28, 142.98, 141.09, 138.77, 134.34, 134.32, 130.43, 130.21, 129.19, 128.69, 128.64, 128.21, 127.99, 127.86, 122.53, 121.53, 120.22, 118.12, 114.81. ESI/MS (*m/z*): $[M+H]^+$ 459.17. Anal. calcd. for $C_{34}H_{30}N_2O_7$: C, 78.59; H, 4.84; N, 6.11. Found: C, 78.62; H, 4.80; N, 6.10.

*(E)-1-(3-Hydroxy-4-((E)-(4-((E)-3-(2-methoxyphenyl)-acryloyl)phenyl)diazenyl)phenyl)-3-(2-methoxyphenyl)-prop-2-en-1-one (**5**)*

Dark red solid, FT-IR (KBr, cm^{-1}): 3396, 3044, 1673, 1533. ^{1}H NMR (200 MHz, $CDCl_3$): δ 8.16 (d, J = 8.6, 1H, ArH), 8.02 (m, 2H, ArH), 7.97 (m, 1H, ArH), 7.82–7.89 (m, 3H, ArH), 7.80 (d, J = 7.8, 1H, ArH), 7.61 (B part of AB system, J = 15.0, 1H), 7.41–7.44 (m, 2H, ArH), 7.25 (A part of AB system, J = 15.0, 1H), 7.10–7.12 (m, 2H, ArH), 7.01–7.05 (m, 2H, ArH), 6.96 (s, 1H, ArH), 3.86 (s, OCH_3, 3H), 3.74 (s, OCH_3, 3H). ^{13}C NMR (50 MHz, $CDCl_3$): δ 195.19, 187.94, 161.98, 158.75, 158.48, 154.57, 142.93, 141.99, 139.36, 139.22, 138.53, 133.07, 132.85, 129.01, 128.59, 123.67, 123.15, 122.88, 122.01, 121.19, 119.97, 112.23, 112.21, 61.15, 51.05. ESI/MS (*m/z*): $[M+H]^+$ 519.18. Anal. calcd. for $C_{32}H_{26}N_2O_5$: C, 74.12; H, 5.05; N, 5.40. Found: C, 74.10; H, 5.08; N, 5.38.

*(E)-1-(3-Hydroxy-4-((E)-(4-((E)-3-(3-methoxyphenyl)-acryloyl)phenyl)diazenyl)phenyl)-3-(3-methoxyphenyl)-prop-2-en-1-one (**6**)*

Dark red solid, FT-IR (KBr, cm^{-1}): 3258, 3045, 1657, 1593. ^{1}H NMR (200 MHz, $CDCl_3$): δ 8.03 (m, 2H, ArH), 7.98 (m, 2H, ArH), 7.87 (d, J = 8.6, 1H, ArH), 7.82 (d, J = 8.6, 1H, ArH), 7.42 (A part of AB system, J = 16.4, 1H), 7.80 (B part of AB system, J = 16.4, 1H), 7.30 (m, 2H, ArH), 7.22 (m, 2H, ArH), 7.13 (s, 2H, ArH), 6.99–7.10 (m, 2H, ArH), 6.90 (s, 1H, ArH), 3.86 (s, OCH_3, 3H), 3.74 (s, OCH_3, 3H). ^{13}C NMR (50 MHz, $CDCl_3$): δ 196.11, 189.99, 160.34, 159.82,

159.76, 154.74, 145.40, 144.25, 143.71, 140.53, 139.04, 135.91, 135.19, 129.90, 129.53, 127.99, 129.90, 122.86, 121.92, 121.18, 121.07, 116.54, 115.21, 113.45, 55.32, 55.20. ESI/MS (*m/z*): $[M+H]^+$ 519.18. Anal. calcd. for $C_{32}H_{26}N_2O_5$: C, 74.12; H, 5.05; N, 5.40. Found: C, 74.10; H, 5.07; N, 5.38.

*(E)-1-(3-Hydroxy-4-((E)-(4-((E)-3-(4-methoxyphenyl)-acryloyl)phenyl)diazenyl)phenyl)-3-(4-methoxyphenyl)-prop-2-en-1-one (**7**)*
Dark red solid, FT-IR (KBr, cm^{-1}): 3201, 3049, 1655, 1571. ^1H NMR (200 MHz, DMSO-d_6): δ 8.20 (d, $J = 7.4$, 2H, ArH), 8.16 (d, $J = 7.4$, 2H, ArH), 7.81 (m, 1H, ArH), 7.78 (m, 1H, ArH), 7.76 (B part of AB system, $J = 15.2$, 1H), 7.63 (d, $J = 7.8$, 2H, ArH), 7.31 (A part of AB system, $J = 15.2$, 1H), 7.63 (d, $J = 7.8$, 2H, ArH), 7.18 (s, 1H, ArH), 6.98 (d, $J = 7.8$, 2H, ArH), 3.80 (s, OCH_3, 3H), 3.75 (s, OCH_3, 3H). ^{13}C NMR (50 MHz, DMSO-d_6): δ 195.21, 188.91, 162.20, 162.07, 154.83, 145.16, 144.40, 143.16, 142.66, 139.63, 131.66, 131.20, 130.43, 131.20, 130.18, 127.92, 127.54, 126.42, 123.01, 121.02, 120.10, 118.65, 115.12, 56.08. ESI/MS (*m/z*): $[M+H]^+$ 519.17. Anal. calcd. for $C_{32}H_{26}N_2O_5$: C, 74.12; H, 5.05; N, 5.40. Found: C, 74.10; H, 5.07; N, 5.38.

*(E)-3-(2,3-Dimethoxyphenyl)-1-(4-((E)-(4-((E)-3-(2,3-dimethoxyphenyl)acryloyl)-2-hydroxyphenyl)diazenyl)-phenyl)prop-2-en-1-one (**8**)*
Dark red solid, FT-IR (KBr, cm^{-1}): 3200, 3035, 1654, 1573. ^1H NMR (200 MHz, $CDCl_3$): δ 8.02 (d, $J = 7.4$, 2H, ArH), 7.98 (d, $J = 7.4$, 2H, ArH), 7.85 (d, $J = 8,6$, 1H, ArH), 7.81 (d, $J = 8,6$, 1H, ArH), 7.76 (s, 1H, ArH), 7.61 (B part of AB system, $J = 15.0$, 1H), 7.25 (A part of AB system, $J = 15.0$, 1H), 7.09 (m, 2H, ArH), 6,99 (m, 2H, ArH), 6.91 (m, 2H, ArH), 3.88 (s, OCH_3, 6H), 3.82 (s, OCH_3, 3H), 3.71 (s, OCH_3, 3H). ^{13}C NMR (50 MHz, $CDCl_3$): δ 196.22, 190.20, 160.18, 154.45, 153.15, 153.01, 148.93, 148.65, 143.90, 140.87, 140.10, 139.28, 138.87, 129.53, 129.04, 128.86, 128.65, 124.27, 123.23, 122.90, 120.92, 119.55, 119.13, 118.60, 115.32, 114.30, 61.46, 61.37, 55.85, 55.79. ESI/MS (*m/z*): $[M+H]^+$ 579.20. Anal. calcd. for $C_{34}H_{30}N_2O_7$: C, 70.58; H, 5.23; N, 4.84. Found: C, 70.60; H, 5.21; N, 4.80.

*(E)-3-(2,4-Dimethoxyphenyl)-1-(4-((E)-(4-((E)-3-(2,4-dimethoxyphenyl)acryloyl)-2-hydroxyphenyl)diazenyl)-phenyl)prop-2-en-1-one (**9**)*
Dark red solid, FT-IR (KBr, cm^{-1}): 3211, 3040, 1656, 1579. ^1H NMR (200 MHz, $CDCl_3$): δ 8.08 (B part of AB system, $J = 15.0$, 1H), 8.01 (d, $J = 7.8$, 1H, ArH), 7.97 (d, $J = 7.8$, 1H, ArH), 7.93 (bs, 2H, ArH), 7.86 (d, $J = 7.8$, 1H, ArH), 7.82 (d, $J = 7.8$, 1H, ArH), 7.72 (s, 1H, ArH), 7.55 (A part of AB system, $J = 15.0$, 1H), 7.18 (bs, 2H, ArH), 6.54 (d, $J = 7.8$, 1H, ArH), 6.41 (bs, 1H, ArH), 6.46 (s, 1H, ArH), 6.38 (s, 1H, ArH), 3.87 (s, OCH_3, 3H), 3.85 (s, OCH_3, 3H), 3.78 (s, OCH_3, 3H), 3.70 (s, OCH_3, 3H). ^{13}C NMR (50 MHz, $CDCl_3$): δ 196.77, 190.71, 163.25, 163.19, 160.52, 160.30, 160.28, 154.42, 143.65, 141.29, 141.72, 140.67, 139.67, 131.22, 129.62, 129.34, 126.15, 125.23, 122.85, 119.94, 116.80, 116.50, 115.22, 105.73, 105.41, 98.30, 97.85, 55.49. ESI/MS (*m/z*): $[M+H]^+$ 579.20. Anal. calcd. for $C_{34}H_{30}N_2O_7$: C, 70.58; H, 5.23; N, 4.84. Found: C, 70.59; H, 5.21; N, 4.80.

*(E)-3-(2,5-Dimethoxyphenyl)-1-(4-((E)-(4-((E)-3-(2,5-dimethoxyphenyl)acryloyl)-2-hydroxyphenyl)diazenyl)-phenyl)prop-2-en-1-one (**10**)*
Dark red solid, FT-IR (KBr, cm^{-1}): 3395, 3044, 1672, 1532. ^1H NMR (200 MHz, $CDCl_3$): δ 8.21 (d, $J = 8.6$, 1H, ArH), 7.96 (d, $J = 8.6$, 2H, ArH), 7.91 (d, $J = 8.6$, 1H, ArH), 7.81 (d, $J = 8.6$, 2H, ArH), 7.64 (B part of AB system, $J = 16.4$, 1H), 7.31 (A part of AB system, $J = 16.4$, 1H), 7.24 (bs, 2H, ArH), 7.04–7.06 (bs, 3H, ArH), 6.96 (bs, 2H, ArH), 3.81 (s, OCH_3, 3H), 3.77 (s, OCH_3, 3H), 3.69 (s, OCH_3, 3H), 3.63 (s, OCH_3, 3H). ^{13}C NMR (50 MHz, $CDCl_3$): δ 195.92, 189.09, 162.21, 154.88, 153.89, 153.81, 153.49, 153.16, 143.20, 142.35, 139.46, 139.30, 138.32, 129.04, 123.94, 123.13, 122.45, 121.26, 119.17, 119.04, 113.71, 113.21, 113.02, 56.82, 56.73, 56.37, 56.14. ESI/MS (*m/z*): $[M+H]^+$ 579.21. Anal. calcd. for $C_{32}H_{26}N_2O_5$: C, 70.58; H, 5.23; N, 4.84. Found: C, 70.60; H, 5.25; N, 4.82.

*(E)-3-(3,4-Dimethoxyphenyl)-1-(4-((E)-(4-((E)-3-(3,4-dimethoxyphenyl)acryloyl)-2-hydroxyphenyl)diazenyl)-phenyl)prop-2-en-1-one (**11**)*
Dark red solid, FT-IR (KBr, cm^{-1}): 3191, 3037, 1650, 1586. ^1H NMR (200 MHz, DMSO-d_6): δ 8.25 (d, $J = 8.0$, 2H, ArH), 7.84 (d, $J = 8.0$, 2H, ArH), 7.80 (bs, 1H, ArH), 7.74 (B part of AB system, $J = 15.0$, 1H), 7.54 (bs, 1H, ArH), 7.32 (m, 2H, ArH), 7.10 (bs, 1H, ArH), 7.38 (A part of AB system, $J = 15.0$, 1H), 7.05 (bs, 1H, ArH), 7.01 (m, 2H, ArH), 6,96 (s, 1H, ArH), 3.85 (s, OCH_3, 3H), 3.82 (s, OCH_3, 3H), 3.77 (ss, OCH_3, 3H), 3.72 (s, OCH_3, 3H). ^{13}C NMR (50 MHz, DMSO-d_6): δ 195.26, 188.91, 162.13, 154.90, 152.12, 152.01, 149.70, 149.64, 145.71, 145.03, 143.17, 142.88, 139.67, 130.45, 128.01, 127.77, 126.60, 124.96, 124.10, 123.03, 120.80, 118.91, 118.60, 115.04, 112.19, 111.23, 56.41, 56.22. ESI/MS (*m/z*): $[M+H]^+$ 579.20. Anal. calcd. for $C_{34}H_{30}N_2O_7$: C, 70.58; H, 5.23; N, 4.84. Found: C, 70.55; H, 5.20; N, 4.80.

*(E)-3-(3,5-Dimethoxyphenyl)-1-(4-((E)-(4-((E)-3-(3,5-dimethoxyphenyl)acryloyl)-2-hydroxyphenyl)diazenyl)-phenyl)prop-2-en-1-one (**12**)*
Dark red solid, FT-IR (KBr, cm^{-1}): 3179, 3031, 1655, 1589. ^1H NMR (200 MHz, DMSO-d_6): δ 8.25 (bs, 1H, ArH), 7.91 (bs, 2H, ArH), 7.83 (bs, 1H, ArH), 7.79 (bs, 1H, ArH), 7.71 (B part of AB system, $J = 15.2$, 1H), 7.33 (s, 1H, ArH), 7.08 (bs, 2H, ArH), 7.10 (bs, 2H, ArH), 6.95 (s, 1H, ArH), 6.87 (s, 1H, ArH), 6.59 (A part of AB system, $J = 15.2$, 1H), 3.78 (s, OCH_3, 3H), 3.69 (s, OCH_3, 3H). ^{13}C NMR (50 MHz, DMSO-d_6): δ 195.33, 189.13, 162.27, 161.40, 154.95, 145.28, 144.08, 143.23, 142.10, 139.30, 137.21, 137.06, 130.64, 129.26, 123.10, 121.56, 118.83, 115.21, 107.51, 106.98, 103.73, 56.15, 56.03. ESI/MS (*m/z*): $[M+H]^+$ 579.20. Anal. calcd. for $C_{34}H_{30}N_2O_7$: C, 70.58; H, 5.23; N, 4.84. Found: C, 70.54; H, 5.19; N, 4.79.

*(E)-1-(3-Hydroxy-4-((E)-(4-((E)-3-(2,3,4-trimethoxyphenyl)-acryloyl)phenyl)diazenyl)phenyl)-3-(2,3,4-trimethoxyphenyl)prop-2-en-1-one (**13**)*
Dark red solid, FT-IR (KBr, cm^{-1}): 3153, 3028, 1635, 1579. ^1H NMR (200 MHz, $CDCl_3$): δ 8.04 (d, $J = 7.0$, 2H, ArH), 7.88

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(bs, 2H, ArH), 7.87 (bs, 1H, ArH), 7.85 (bs, 1H, ArH), 7.74 (B part of AB system, d, $J = 15.6$, 1H), 7.52 (s, 1H, ArH), 7.41 (m, 1H), 6.75 (m, 2H, ArH), 6.58 (m, 2H, ArH), 3.83 (s, OCH_3, 3H), 3.72 (s, OCH_3, 3H), 3.68 (s, OCH_3, 3H). ^{13}C NMR (50 MHz, $CDCl_3$): δ 196.52, 190.32, 160.15, 155.95, 154.43, 153.83, 153.56, 143.77, 142.31, 142.17, 140.75, 139.93, 139.54, 129.43, 126.87, 124.08, 123.45, 122.85, 121.70, 121.42, 120.92, 118.43, 115.20, 107.58, 61.56, 61.43, 60.92, 56.07. ESI/MS (*m/z*): $[M+H]^+$ 639.23. Anal. calcd. for $C_{36}H_{34}N_2O_9$: C, 67.70; H, 5.37; N, 4.39. Found: C, 67.71; H, 5.33; N, 4.38.

(E)-1-(3-Hydroxy-4-((E)-(4-((E)-3-(3,4,5-trimethoxyphenyl)-acryloyl)phenyl)diazenyl)phenyl)-3-(3,4,5-trimethoxyphenyl)prop-2-en-1-one ***(14)***

Dark red solid, FT-IR (KBr, cm^{-1}): 3201, 3051, 1656, 1580. ^{1}H NMR (200 MHz, $CDCl_3$): δ 8.31 (m, 2H, ArH), 8.20 (m, 2H, ArH), 7.88 (bs, 1H, ArH), 7.84 (bs, 1H, ArH), 7.72 (s, 1H, ArH), 7.65 (B part of AB system, d, $J = 15.6$, 1H), 7.31 (A part of AB system, d, $J = 15.6$, 1H), 7.0 (m, 2H, ArH), 6.95 (m, 2H, ArH), 3.76 (s, OCH_3, 6H), 3.71 (s, OCH_3, 3H). ^{13}C NMR (50 MHz, $CDCl_3$): δ 192.75, 186.32, 159.55, 152.28, 151.12, 142.99, 142.17, 140.56, 137.82, 137.62, 136.85, 128.20, 127.85, 125.60, 120.44, 119.13, 118.28, 116.01, 112.44, 104.53, 104.04, 58.19, 54.11, 53.96. ESI/MS (*m/z*): $[M+H]^+$ 639.22. Anal. calcd. for $C_{36}H_{34}N_2O_9$: C, 67.70; H, 5.37; N, 4.39. Found: C, 67.71; H, 5.33; N, 4.38.

CA inhibition

Enzyme activity was determined spectrophotometrically by following the change in absorbance at 348 nm of 4-nitrophenylacetate to 4-nitrophenol over a period of 3 min at 25°C [38]. The enzymatic reaction contained 1.4 mL 0.05 M Tris-SO_4 buffer (pH 7.4), 1 mL 3 mM 4-nitrophenylacetate, 0.5 mL H_2O, and 0.1 mL enzyme solution, in a total volume of 3.0 mL [42–45]. Inhibitory effects of compounds **3–14** were compared with AZA. Different inhibitor concentrations were used and all compounds were tested in triplicate at each concentration used. Control cuvette activity was acknowledged as 100% in the absence of inhibitor. An Activity % − [Inhibitor] graph was drawn for each inhibitor [46]. The curve-fitting algorithm allowed for obtaining the IC_{50} values, working at the lowest concentration of substrate of 0.15 mM, from which K_I values were calculated [42–45]. The catalytic activity of these enzymes was calculated from Lineweaver–Burk plots, as reported previously [46], and represent the mean from at least three different determinations. The CA I and II isoenzymes used here were purified from human blood as previously described [39–41, 47, 48].

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