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9,10-Dibromo-*N*-aryl-9,10-dihydro-9,10-[3,4]epipyrrolo-anthracene-12,14-diones: Synthesis and Investigation of Their Effects on Carbonic Anhydrase Isozymes I, II, IX, and XII

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N-substituted maleimides were synthesized from maleic anhydride and primary amines. 1,4-Dibromo-dibenzo[e,h]bicyclo-[2,2,2]octane-2,3-dicarboximide derivatives (**4a–f**) were prepared by the [4+2] cycloaddition reaction of dibromoanthracenes with the *N*-substituted maleimide derivatives. The carbonic anhydrase (CA, EC 4.2.1.1) inhibitory effects of the new derivatives were assayed against the human (h) isozymes hCA I, II, IX, and XII. All tested bicyclo dicarboximide derivatives exhibited excellent inhibitory effects in the nanomolar range, with K_i values in the range of 117.73–232.87 nM against hCA I and of 69.74–111.51 nM against hCA II, whereas they were low micromolar inhibitors against hCA IX and XII.

Keywords: Carbonic anhydrase / Enzyme inhibition / Enzyme purification / Isoenzyme

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Introduction

N-substituted cyclic imides may be used for regulating serotonin levels in humans [1]. Such derivatives show antidepressant effects, being used for the treatment of disorders such as anxiety or as analgesics [2–4]. The bicylo anthracene scaffold containing agent, 3-(9,10-dihydro-9,10-ethanoanthracene-9-yl) *N*-methylpropylamine (maprotiline) also shows antidepressant properties [5] and imides incorporating this skeleton act as glucocorticoid receptor modulators [6].

In recent years, fungal infections emerged as a serious health problem with few new drugs for the treatment of resistant infections available [7]. Many of the currently used drugs have adverse effects or should be used for long period in order to be effective [8]. Several studies showed the capability of maleimide derivatives to be used as the antifungal reagents [9]. Few studies on the synthesis of 1,4-dibromo-dibenzo[e,h]dicyclo-[2,2,2]-octane-2,3-dicarboximide derivatives are available. For the synthesis of such molecules, the 1,4-dibromo-dibenzo[e,h]-bicyclo[2,2,2]octane-2,3-dicarboxymide structure may be

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obtained by using 9,10-dibromoanthracene and malemide as starting materials, by a [4+2] cycloaddition reaction. *N*-Alkyl-1,4-dibromo-dibenzo[e,h]bicyclo[2,2,2]octane-2,3-dicarboximide derivatives were obtained by this reaction [1, 10].

In this study, 9,10-dibromoanthracene and maleic anhydride derivatives were synthesized according to the literature procedures. These dicarboximides derivatives underwent cycloaddition reaction, leading to the tetracyclic 9,10-dibromoanthracene maleimides with high yields (Scheme 1).

Enzymes, biological molecules responsible for thousands of metabolic processes, are synthesized by living cells and speed up chemical reactions during the metabolism of living organisms [11]. Carbonic anhydrases (CA; carbonate hydrolase, EC 4.2.1.1) are a widely spread super-family of zinc metalloenzymes that catalyze the reversible hydration of carbon dioxide (CO_2) and water (H_2O) to bicarbonate (HCO_3^-) and proton (H^+) [12–14].

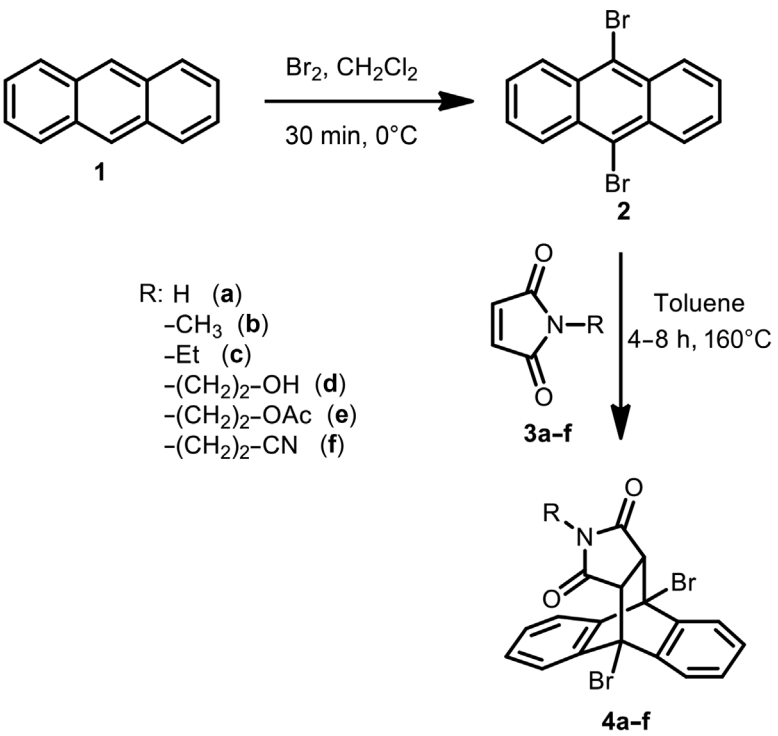
$$CO_2 + H_2O \overset{CA}{\Leftrightarrow} H_2CO_3 \Leftrightarrow HCO_3^- + H^+$$

They play important roles in some pathological and physiological processes including acid-based balance, bone resorption, electrolyte secretion, CO_2 and ion transport, respiration, ureagenesis, lipogenesis, and gluconeogenesis [15–17].

Up to now, six different genetically distinct CA families are known: α-, β-, γ-, δ-, ζ-, and η-CAs. The α-carbonic anhydrases (α-CAs) are widespread metalloenzymes in higher vertebrates including humans. α-, β-, and δ-CAs use Zn^{2+} ions at the active site, the γ-CAs are probably Fe^{2+} enzymes, but they are active also with bound Zn^{2+} or Co^{2+} ions, whereas the ζ-class uses Cd^{2+} or Zn^{2+} to perform the physiologic reaction catalysis [15, 18, 19]. Up to now, 16 CA isoforms from α-CA have been identified in mammals that differ in subcellular localization, catalytic activity, and susceptibility to different classes of inhibitors [13, 20].

The α-CAs exist in vertebrates, protozoa, algae, and cytoplasm of green plants and in some bacteria [21]. In humans, α-CAs are present in various tissues such as the gastrointestinal tract, the reproductive tract, kidneys, the nervous system, skin and eyes, lungs, among others. Of these isoenzymes, CA I–III, VII, and XIII are cytosolic, CA IV, IX, XII, and XIV are membrane bound, CA VA and VB are mitochondrial, and CA VI is secreted in saliva. Also, the recently reported CA XV isoform is not expressed in humans or in other primates. Three catalytic forms (CARP VIII, X, and XI) are also known and called CA-related proteins (CARPs) [22–24].

An enzyme inhibitor is a substance that binds to an enzyme and decreases its activity. CA inhibitors (CAIs) are a class of pharmaceuticals used as diuretics, antiepileptics, antiglaucoma agents, and in the management of mountain sickness, gastric, and duodenal ulcers, osteoporosis, neurological disorders including epilepsy, and in the treatment of Alzheimer's disease [25–27]. The sulfonamides are the classical inhibitors of CA [15, 28–31]. They have been widely used for almost 60 years as diuretic or systemically acting antiglaucoma



Scheme 1. A new method for the synthesis of *N*-alkyl-1,4-dibromo-dibenzo[e,h]bicyclo[2,2,2]octane-2,3-dicarboximide derivatives (**4a–f**).

drugs, since the introduction of acetazolamide (AAZ) in clinical use in 1954 [18, 25, 26, 32]. However, AAZ and the other clinically used sulfonamides/sulfamate CAIs [15, 18, 25] showed a range spectrum of undesired side effects [15, 25] motivating the continuous search of novel such agents with a selective inhibition profile against the desired isoforms [33–35]. Herein, we report the synthesis of a new method for synthesis of 1,4-dibromo-dibenzo[e,h]bicyclo[2,2,2]-octane-2,3-dicarboximide derivatives and determined their effects on two cytosolic carbonic anhydrase isoforms (hCA I and II), cancer-associated transmembrane carbonic anhydrase isoforms (hCA IX and XII).

Results and discussion

Chemistry

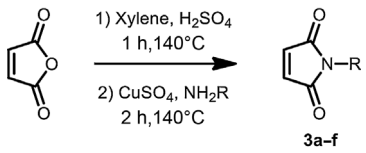
9,10-Dibromoanthracene (**2**) was prepared in accordance with literature procedures. The reaction occurred when a concentrated bromine solution was added to anthracene (**1**), which acted as solvent. Ambient temperature for brominating of anthracene (**1**) is very important. Due to changes in temperature, the different isomers are formed. Therefore, 9,10-dibromoanthracene (**2**, 99%) was obtained from anthracene by bromination at 0°C.

Maleimide derivatives (**3a–f**) were synthesized by the addition of primary amines and maleic anhydride to the reaction mixture. These derivatives (**3a–f**) were checked with ^{1}H and ^{13}C spectroscopy and the spectra were compared with the literature data (Table 1). All maleimide derivatives listed in Table 1 were reacted with 9,10-dibromoanthracene (**2**) in toluene. For example, as result of cycloaddition reactions, 1*H*-pyrrole-2,5-dione (**3a**), 1-methyl-1*H*-pyrrole-2,5-dione (**3b**), 1-ethyl-1*H*-pyrrole-2,5-dione (**3c**), 1-(2-hydroxyethyl)-1*H*-pyrrole-2,5-dione (**3d**), 2-(2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)ethyl acetate (**3e**), 3-(2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)propanenitrile (**3f**) molecules were converted to 1,4-dibromo-dibenzo[e,h]-bicyclo[2,2,2]octane-2,3-dicarboxymide (**4a**, 95%), *N*-methyl-1,4-dibromo-dibenzo[e,h]bicyclo[2,2,2]octane-2,3-dicarboxymide (**4b**, 95%), *N*-ethyl-1,4-dibromo-dibenzo[e,h]-bicyclo[2,2,2]octane-2,3-dicarboxymide (**4c**, 92%), *N*-(2-hydroxyethyl)-1,4-dibromo-dibenzo[e,h]bicyclo[2,2,2]octane-2,3-dicarboxymide (**4d**, 94%), *N*-(ethylacetate)-1,4-dibromo-dibenzo[e,h]bicyclo[2,2,2]octane-2,3-dicarboximide (**4e**, 94%), *N*-(propanenitrile)-1,4-dibromo-dibenzo[e,h]bicyclo-[2,2,2]octane-2,3-dicarboxymide (**4f**, 95%) (Table 2).

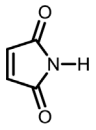
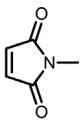
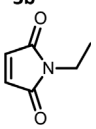
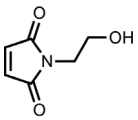
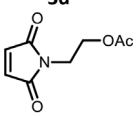
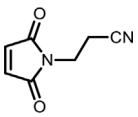
Biochemistry (CA inhibition)

All the synthesized compounds were assayed to establish their inhibitory effect against the ubiquitous off-target isoforms, CA I and II by esterase method, and the cancer-related ones, CA IX and XII by a stopped-flow, CO_2 hydrase assay method [36]. The inhibition data of the new bicyclo dicarboximide derivatives (**4a–f**) reported here are shown in Table 3.

Table 1. Synthesis of maleimide derivatives (3a–f).[a)]



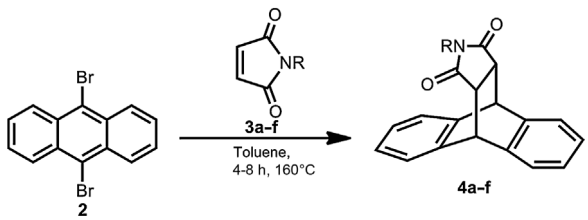
3a–f

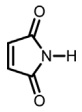
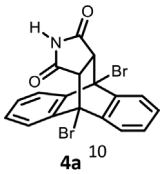
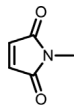
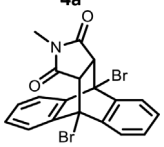
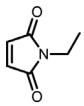
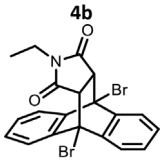
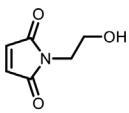
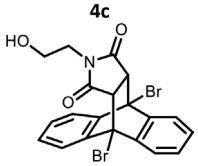
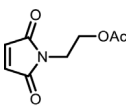
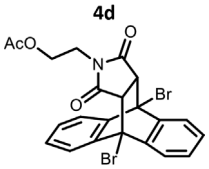
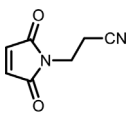
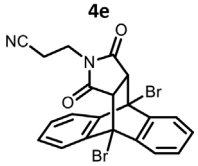
Entry	R	Products[b)]	Yield[c)] (%)
1	H	 **3a** [1,10]	95
2	CH_3	 **3b** [11,12]	90
3	$-C_2H_5$	 **3c** [12]	85
4	$-(CH_2)_2OH$	 **3d** [13]	88
5	$-(CH_2)_2OCOCH_3$	 **3e** [13,14]	88
6	$-(CH_2)_2CN$	 **3f** [15]	85

[a)] Reaction condition: all reactions were performed using xylene (54.0 mmol), sulfuric acid (15.0 mmol), maleic anhydrite (30.0 mmol), copper sulfate (0.24 mmol), and alkyl amine (45.0 mmol) at 140°C. [b)] The synthesis and NMR data of **3a–f** compounds in the literature; [c)] isolated yield.

The known human CA isozymes belong to the α-CA family present in mammals and the most common ubiquitous isoforms are the cytosolic hCA I and II. hCA I is ubiquitously expressed in the body, and can be found in high concentrations in the blood and gastrointestinal tract [37]. It was reported that if the inhibition constant (K_i) of a tested compound was less than 50 μM ($K_i > 50$ μM), it was considered low inhibition and this inhibitor was accepted to be inactive against hCA I [37, 38]. Our results indicate that new

Table 2. **[4+2] Cycloaddition reactions of 9,10-dibromoanthracene.**[a)]



Entry	Substrate	Product[b)]	Yield[c)] (%)
1	 **3a**	 **4a** [10]	95
2	 **3b**	 **4b**	95
3	 **3c**	 **4c**	92
4	 **3d**	 **4d**	94
5	 **3e**	 **4e**	94
6	 **3f**	 **4f**	95

[a)] All reactions were performed in toluene (10.0 mL) using 9,10-dibromoanthracene (10.0 mmol, 3.36 g) and maleimide derivatives (10.0 mmol) at 160°C. [b)] The synthesis and NMR data of **4a** compound in the literature; [c)] isolated yield.

bicyclo dicarboximide derivatives (**4a–f**) had effective inhibition profile against slow cytosolic isoform hCA I, and cytosolic dominant rapid isozyme hCA II. The cytosolic isoenzyme hCA I was effectively inhibited by all of the newly synthesized bicyclo dicarboximide derivatives (**4a–f**) with inhibition constants in the low nanomolar range. K_i values are ranging from 117.73 to 232.87 nM against hCA I. Also, the average K_i values of these new synthesized bicyclo dicarboximide derivatives (**4a–f**) for hCA I was found as 145.56 nM. On the other hand, the most known and clinically established CA

Table 3. Human carbonic anhydrase isoenzymes I, II, IX, and XII inhibition values of some new bicyclo dicarboximide derivatives (4a–f).

Compounds	hCA I (nM)	K_i hCA II (nM)	hCA IX (μM)	hCA XII (μM)
4a	125.24	97.01	>50	9.00
4b	132.81	94.78	>50	10.10
4c	117.73	101.39	>50	7.40
4d	232.87	111.51	>50	10.60
4e	126.81	69.74	>50	>50
4f	137.90	93.86	11.4	>50
AAZ[a)]	184.30	61.10	0.025	0.006

[a)] Acetazolamide (AAZ) was used as a standard inhibitor for all CAs investigated here.

inhibitor is AAZ, and this sulfonamide derivative was used as positive control in this study. For compound **4c**, which has carbonyl, acetate, and dibromide groups, is showed effective hCA I inhibitory property. This was the best inhibitor of this isoform among the reported bicyclo dicarboximide derivatives (**4a–f**). All of the new synthesized bicyclo dicarboximide derivatives except of compound **4d** demonstrated effective hCA I inhibition effect than the clinically used AAZ (184.30 nM). These results clearly showed that many of the investigated compounds were more effective or similar to AAZ for the inhibition of this isoform.

It was reported that defective bone resorption and a general failure of bone remodeling characterize osteopetrosis, a rare disease that produces dense, brittle bone, and can be caused by a hereditary deficiency in hCA II [39]. Against the physiologically dominant isoform hCA II, these bicyclo dicarboximide derivatives (**4a–f**) demonstrated K_i values of 69.74–111.51 nM (Table 3). Compound **4e**, with acetate, dicarbonyl, and dibromine groups, was the best hCA II inhibitor (K_i: 69.74 nM). However, the average K_i values of newly synthesized bicyclo dicarboximide derivatives (**4a–f**) were found to be 94.78 nM for hCA II. This average result is lower than that of hCA I (145.56 nM). This result showed that newly synthesized bicyclo dicarboximide derivatives (**4a–f**) had higher inhibition affinity to CA II isoenzyme than that of CA I isoenzyme.

hCA IX and XII isoforms have been validated as targets of antitumor agents [40, 41]. In fact, the bicarbonate (HCO_3^-) ions produced at the extracellular surface by CA IX is transferred into the cytosol to regulate intracellular pH while proton (H^+) produced by CA IX contribute to diminish extracellular pH, causing matrix breakdown and metastasis and tumor invasion.

Same newly synthesized bicyclo dicarboximide derivatives (**4a–f**) were also effective as inhibitors of the two transmembrane isoforms, hCA IX and XII; although in the micromolar, not nanomolar range (Table 3). Indeed, against hCA IX bicyclo dicarboximide derivative **4f** showed moderate inhibition constant (11.4 μM), the other derivatives (**4a–e**) showed no inhibitory effects up to 50 μM in the assay system. It may be observed that the presence of acetate, carbonyl, or bromide moieties in these new compounds leads to derivatives with rather similar activity profile, and the main factor influencing CA inhibitory effects is the substitution pattern at the phenyl ring. It was well-known that the compound with these groups (acetate, carbonyl, and bromide) is in favor of inhibition of CA isoenzymes [40].

On the other hand, against hCA XII, some new bicyclo dicarboximide derivatives (**4a–d**) reported here also show interesting inhibitory effects, with inhibition constants ranging between 7.40 and 10.60 μM (Table 3). The best hCA XII inhibitor is again only with $-NCH_2CH_3$, carbonyl moieties, and two Br substituent (compound **4c**). These encouraging biological results demonstrated that this series of bicyclo dicarboximide derivatives (**4a–f**) could represent an important starting point for the development of new antiglaucoma and anticancer agents.

Recently, the role of CAIs as anticancer agents is rapidly gaining popularity [42, 43] due to the observation that membrane bound hCA isozymes hCA IX and XII are overexpressed in hypoxic tumors [44–49]. hCA IX and XII isoforms are overexpressed both in primary and in metastatic cell lines of hypoxic tumors and are innovative targets for cancer diagnosis and treatment [40]. Recently, numerous inhibitors of CA IX have been developed for prevention of tumor acidification processes and re-establishment of a more normal pH might lead to regression of the tumor. These inhibitors especially were used in combination with classical anticancer drugs. Also, it has been demonstrated that CA IX and XII isozymes are prominently associated with and overexpressed in many tumors [44]. They are involved in important processes connected with cancer progression and response to therapy [50, 51]. Firstly, the CA IX was found to be associated with cancers in literature [52]. The tumor-associated isoform hCA IX is highly overexpressed in many cancer types by the hypoxia inducible factor-1α (HIF-1α) cascade. hCA IX is also expressed in cancer-related fibroblasts in prostate carcinoma cells causing major motility and survival [40, 53]. It has been demonstrated that the inhibition of CA IX supports anti-VEGF therapy [40, 54]. Also, CA XII was subsequently shown to be co-expressed with CA IX in some tumor tissues. It was additionally found in a wider

range of normal tissues [55]. As distinct from other CA isoforms, it has one polypeptide chain comprising only the catalytic domain, CA IX is a multidomain, transmembrane protein with a more complex organization. This isoform has (i) a short signal peptide; (ii) a small transmembrane segment; (iii) a small intracytosolic tail with an unknown function; (iv) the extracellular catalytic domain, which shows high sequence homology to the catalytic domain of other α-CAs [50, 52]; and (v), it has also a proteoglycan-like domain unique to CA IX, which is crucial to the cell-adhesion processes in which this protein is involved [52]. Many experiments have been demonstrated that the protein constructs lacking either the proteoglycan-like domain or the catalytic domain in order to understand the relevance of the various domains to its function and role in tumorigenesis [52, 56–60]. This cancer-associated CA isoform is susceptible to inhibition by a large spectrum of chemical such as phenols [61] anions, and sulfonamides or sulfamates, as similar to all other α-CA isoenzymes [25, 60]. The inhibitors coordinate the Zn^{2+} ion directly in the active-site cavity of CA and join in various other favorable interactions with amino acids situated in both the hydrophilic and the hydrophobic halves of the active site [25, 55].

Conclusion

A new series of *N*-alkyl-1,4-dibromo-dibenzo[e,h]bicyclo [2,2,2]octane-2,3-dicarboxymide derivatives (**4a–f**) were designed, synthesized, and assayed against four human carbonic anhydrases: CA IX and XII that are the two tumor-associated isozymes, and CA I and II, which represent the most common off-targets for the development of selective anticancer CA inhibitors. Low nanomolar levels of K_i values were observed for all compounds, against hCA I and II. All bicyclo dicarboximide derivatives (**4a–d**) had higher affinity for hCA I and II in the nanomolar range, and they all possessed an inhibitory effect selectively on CA IX and XII in the micromolar range. This study clearly showed that the newly synthesized bicyclo dicarboximide derivatives (**4a–d**) possess effective inhibition profiles against two human cytosolic CA isoenzymes (hCA I and II) and two transmembrane cancer-associated CA isoenzymes (hCA IX and XII). These promising results demonstrated that this novel synthesis compounds could represent an innovative and attractive tool for the development of new antitumor agents based on the inhibition of the cancer-related isoforms of human carbonic anhydrase.

Experimental

Materials

Copper (II) sulfate pentahydrate ($CuSO_4.5H_2O$, 99%), sulfuric acid (H_2SO_4, 98%), *m*-xylene (99%), anthracene, bromine (99.5%), toluene (99.8%), maleimide (99%), methyl amine (99%), ethyl amine (99%), ethanolamine (99.5%), 3-hydroxypropanenitrile (97%) were purchased from Sigma–Aldrich and were used without further purification. Other organic compounds; 9,10-dibromoanthracene (99%), 1-methyl-1*H*-pyrrole-2,5-dione (98%), 1-ethyl-1*H*-pyrrole-2,5-dione (98%), 1-(2-hydroxyethyl)-1*H*-pyrrole-2,5-dione (98%), 2-(2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)ethyl acetate (99%), 3-(2,5-dioxo-2,5-dihydro-1*H*-pyrrol-1-yl)propanenitrile (98%) synthesized by the established methods in the literature. ^1H and ^{13}C NMR spectra were recorded on Bruker Avance DPX 400 MHz and Varian AS 400 MHz spectrometers.

The InChI codes and NMR spectra of the new compounds are presented in the Supporting Information.

Chemistry

*Synthesis of 9,10-dibromoanthracene **2***

The 10 mmol of anthracene (**1**) was dissolved in CH_2Cl_2 at 0°C. Later, bromine solution (22 mmol, 1.13 mL Br_2/5 mL CH_2Cl_2) prepared in CH_2Cl_2 was added dropwise for 5 min into the anthracene solution at the same temperature. The reaction was monitored by TLC and completed in 30 min. The solvent (CH_2Cl_2) was evaporated at 30°C and 20 mmHg at the end of the reaction. The product was separated by column chromatography on silica gel with CH_2Cl_2. The yield of the 9,10-dibromoanthracene (**2**) was calculated by ^1H NMR spectra.

*General procedure for the synthesis of maleimide derivatives **3a–f***

A mixture of xylene (54 mmol, 6.8 mL) and sulfuric acid (15 mmol, 1.5 mL) was stirred at 140°C for 1 h. After removal of the water in the reaction medium, the mixture was cooled. Maleic anhydrite (30 mmol, 3 g), copper sulfate (0.24 mmol, 38 mg), and alkyl amine (45 mmol) were added on the cooled mixture and was stirred over the sodium sulfate at 140°C for 2 h. A mixture was made work up a few times over Na_2CO_3 solution using water and ethyl acetate. The solvent was dried and evaporated at 35°C and 20 mmHg at the end of the reaction. The product was separated by column chromatography on silica gel with ethyl acetate/hexane. The yields of the maleimide derivatives (**3a–f**) were calculated by ^1H NMR spectra. The maleimide derivatives were identified by ^1H and ^{13}C NMR spectroscopy in comparison with the literature data (Table 2) [1, 62–67].

*General procedure for the synthesis of N-alkyl-1,4-dibromo-dibenzo[e,h]bicyclo[2,2,2]octane-2,3-dicarboxymide derivatives **4a–f***

The 10 mmol of 9,10-dibromoanthracene (**2**) was dissolved in toluene at room temperature. Next, 10 mmol of maleimide derivatives (**3a–f**) were added into the 9,10-dibromoanthracene solution at the same temperature. The reactions performed in sealed tube were continued for 4 h at 160°C. The solvent (toluene) was evaporated at 40°C and 20 mmHg at the end of the reaction. Finally, the crude residue was directly purified by column chromatography on silica gel using ethyl acetate/hexane as the eluent to separate the synthesized *N*-alkyl-1,4-dibromo-dibenzo[e,h]bicyclo[2,2,2]octane-2,3-dicarboxymide derivatives (**4a–f**). The yields of the *N*-alkyl-1,4-dibromo-

dibenzo[e,h]bicyclo[2,2,2]octane-2,3-dicarboximide derivatives (**4a–f**) were determined by ^{1}H and ^{13}C NMR spectra taken in DMSO-d_6 or $CDCl_3$. The compound **4a** was identified by ^{1}H and ^{13}C NMR spectroscopy in comparison with the literature data (Table 2) [10]. The other maleimide-anthracene derivatives **4b–f** were identified by ^{1}H and ^{13}C NMR spectroscopy (see the Supporting Information for NMR spectral details for **4b–f**).

Biochemistry (CA inhibition)

Both the cytosolic CA isoenzymes (CA I and II) used for inhibition studies of *N*-alkyl-1,4-dibromo-dibenzo[e,h]bicyclo [2,2,2]octane-2,3-dicarboxymide derivatives (**4a–f**) were purified by Sepharose-4B-L tyrosine-sulfanilamide affinity separation technique with a single purification step. Affinity chromatography technique is a method of separating biochemical mixtures based on a highly specific interaction such as that between enzymes and their substrate [68–70]. Sepharose-4B-L tyrosine-sulfanilamide affinity gel was arranged and used according to the previous studies [13, 14]. To this end, the pH of the homogenate was adjusted to 8.7 using solid Tris. Then, the supernatant sample was transferred to a previously prepared affinity column. The quantity of protein in the column effluents was detected spectrophotometrically at 280 nm as previously reported [71–73].

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) describes a technique widely used in biochemistry for separation of biological macromolecule such as proteins and nucleic acids, according to their electrophoretic mobility [38, 74]. SDS-PAGE was applied after purification of both CA isoenzymes. This method was described previously [75–77]. All isoenzymes purities were controlled by SDS-PAGE according to Laemmli's method. A single band was observed for each CA isoenzyme. Briefly, it was performed in acrylamide for the running (10%) and the stacking gel (3%) contained SDS (0.1%), respectively [78–80].

CA isoenzymes activities were determined according to the method of Verpoorte et al. [81] described previously [82–84]. The increase in absorbance at 348 nm of *p*-nitrophenylacetate (NPA) to *p*-nitrophenolate (NP) was recorded during 3 min at 25°C using a spectrophotometer (Shimadzu, UV-VIS spectrophotometer, UVmini-1240). The protein quantity was spectrophotometrically determined at 595 nm during the purification steps according to the Bradford method. As a standard protein, bovine serum albumin (BSA) was used as previously defined. BSA is a serum albumin protein derived from cows and often used as a protein concentration standard in scientific experiments [76, 77, 85]. For the determination of inhibition effect of each newly synthesized bicyclo dicarboximide derivatives, an activity (%)–[bicyclo dicarboximide derivatives] graph was drawn. For calculation of K_i values, three different bicyclo dicarboximide derivatives (**4a–f**) concentrations were used. In this study, as a substrate, NPA was used at five different concentrations. Finally, the Lineweaver–Burk curves were drawn [86]. These drawings calculations for each bicyclo dicarboximide derivatives (**4a–f**) were described in detail previously [87–89].

Both the transmembrane and cancer-associated CA isoenzymes (CA IX and XII) were recombinant isoforms obtained as reported earlier [90–92], and their activity was measured by a stopped-flow CO_2 hydrase procedure [36, 93–96].

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