

IN VITRO INHIBITION OF PURIFIED HUMAN CARBONIC ANHYDRASE I AND II BY NOVEL FLUORENE DERIVATIVES

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In this study, 9-benzylidene-9*H*-fluorene-substituted urea (**5a–p**) and thiourea derivatives (**5q–v**) were synthesized and their inhibitory effects on the activity of human carbonic anhydrase (hCA) I and II were evaluated. hCA I and II were purified from human erythrocytes using a Sepharose 4B-L-tyrosine-sulphanilamide affinity column. All the synthesized compounds inhibited the activity of the hCA I and II isoenzymes. Among the synthesized compounds, **5f** was found to be the most active ($IC_{50} = 21.4 \mu\text{M}$) for inhibition of hCA I and **5s** was the most active ($IC_{50} = 25.3 \mu\text{M}$) for inhibition of hCA II.

Keywords: 9-benzylidene-9*H*-fluorene; urea; thiourea; carbonic anhydrase; inhibition

IN VITRO ИНХИБИЦИЈА НА ПРЕЧИСТЕНА ЧОВЕЧКА КАРБОНСКА АНХИДРАЗА I И II СО НОВИ ФЛУОРЕНСКИ ДЕРИВАТИ

Во оваа студија беа синтетизирани деривати на уреа (**5a–p**) и тиоуреа (**5q–v**) добиени со супституција на 9-безилиден-9*H*-флуорен и беше проценет нивниот инхибиторен ефект врз човечка карбонска анхидраза (hCA) I и II. hCA I и II беа пречистени од човечки еритроцити со употреба на афинитетната колона Sepharose 4B-L-тирозин-сулфаниламид. Сите синтетизирани соединенија ја инхибираа активноста на изoenзимите на hCA I и II. Од синтетизираните соединенија, **5f** се покажа најактивно ($IC_{50} = 21,4 \mu\text{M}$) за инхибиција на hCA I, додека **5s** беше најактивно ($IC_{50} = 25,3 \mu\text{M}$) за инхибиција на hCA II.

Клучни зборови: 9-безилиден-9*H*-флуорен; уреа; тиоуреа; карбонска анхидраза; инхибиција

1. INTRODUCTION

Fluorene-containing compounds have unique chemical behaviours and physical properties due to the unusual geometric structure of fluorene [1]. Fluorene and its derivatives are important materials that are used in organic synthesis, the pharmaceutical and synthetic resin industries, and conductivity research [2–4]. Acetyl-amino-, diacetyl-amino-, amino- and nitro-substituted fluorene compounds increase the biological effects [5, 6] of an inhibitor

of oncogenic tyrosine kinase [7], antimicrobial agents [8] and potent frameshift-type mutagens [9]. Many compounds containing a styryl group have been used as enzyme inhibitors. Some 9-benzylidene-9*H*-fluorene derivatives containing styryl groups may be suitable candidates for CA inhibition [10].

Due to their biological activities, substituted urea and thiourea compounds have potential as chemotherapeutic agents [11, 12], HIV protease inhibitors [13], tyrosinase inhibitors [14, 15], herbicides and antifungal agents [16]. In addition,

recent studies have shown that different urea derivatives have dopamine hydroxylase inhibitory properties, and dopamine is a key precursor of norepinephrine [17]. Also, they are an intermediate product in various total synthesis [18]. Urea derivatives show interesting profiles for the inhibition of several human carbonic anhydrases (hCAs) such as hCA I and II (cytosolic isoforms) and hCA IX and XII (transmembrane, tumour-associated enzymes). The compounds have good inhibitory effects for all these isoforms due to the urea moiety [19].

The metalloenzyme CA (EC 4.2.1.1) catalyses a simple but critically important physiological reaction: members of the CA enzyme family catalyse hydration of CO₂ to yield bicarbonate and a proton. As this reaction is involved in many physiological/pathological processes, there are widespread opportunities for the development of diverse, specific inhibitors for clinical application [20–23].

The active site of most CAs contains a zinc ion (Zn²⁺) that is essential for catalysis. The CA reaction is involved in many physiological and pathological processes, including: respiration and transport of CO₂ and bicarbonate between metabolizing tissues and lungs; pH and CO₂ homeostasis; electrolyte secretion in various tissues and organs; biosynthetic reactions such as gluconeogenesis, lipogenesis and ureagenesis; bone resorption; calcification; and tumourigenicity [24–30]. Many of the CA isoenzymes involved in these processes are important therapeutic targets with the potential to be inhibited and to treat a range of disorders, including oedema, glaucoma, obesity, cancer, epilepsy and osteoporosis [31–35].

In this study, a series of 22 novel 9-benzylidene-9H-fluorene derivatives (**5a–v**) containing urea/thiourea groups were synthesized and their effects on hCA I and II purified from human erythrocytes were evaluated.

2. MATERIALS AND METHODS

Melting points of the synthesized fluorene derivatives were determined by Yanagimoto micro-melting point apparatus and were uncorrected. IR spectra were measured on a Shimadzu Prestige-21 (200 VCE) spectrometer. ¹H and ¹³C NMR spectra were measured on a Varian Infinity Plus spectrometer at 300 and 75 Hz, respectively. ¹H and ¹³C chemical shifts were referenced to the internal deuterated solvent. The elemental analyses were carried out with a Leco CHNS-932 instrument. Flash column chromatography was performed using Merck silica gel 60 (230–400 mesh ASTM). All chemicals were purchased from Merck, Alfa Easer and Sigma-Aldrich.

2.1. Synthesis

of 2-nitro-9-benzylidene-9H-fluorene (3)

2-Nitro-9-benzylidene-9H-fluorene (**3**) was prepared according to the literature [36]. 2-nitrofluorene (4.22 g, 20 mmol) and KOH (3 g, 50 mmol) were stirred in methanol for 30 minutes. Benzaldehyde (2.12 g, 20 mmol) was added and stirred overnight at room temperature. Solvent was evaporated using a rotary evaporator. The mixture was extracted with ethyl acetate (3 × 20 ml). The product was purified by washing with diethyl ether.

2.2. Synthesis

of 2-amino-9-benzylidene-9H-fluorene (4a–b)

2-Amino-9-benzylidene-9H-fluorene was prepared according to the literature [37]. The mixture of 9-benzylidene-2-nitro-9H-fluorene (2.99 g, 10 mmol) and SnCl₂ (11.3 g, 50 mmol) in THF was refluxed for 7 h. THF was removed using a rotary evaporator, and the mixture was extracted with ethyl acetate (3 × 20 ml). At the end of the reaction, two products (including *E*- and *Z*-) were obtained. The products (*E*- and *Z*-) were purified by column chromatography on silica gel using hexane: ethyl acetate (9:1).

2.3. General procedure for the synthesis

of (E or Z)-1-(9-benzylidene-9H-fluoren-2-yl)-3-phenylurea (5a–p)

Isocyanate derivatives (10 mmol) were added to a solution of 2-amino-9-benzylidene-9H-fluorene (*E*- or *Z*-) (2.68 g, 10 mmol) in toluene. The mixture was stirred at 65 °C until precipitation. Toluene was removed using a rotary evaporator, and the product was purified by washing with ethyl ether.

2.4. General procedure for the synthesis

of (E or Z)-1-(9-benzylidene-9H-fluoren-2-yl)-3-phenylthiourea (5q–v)

Isothiocyanate derivatives (10 mmol) were added to a solution of 2-amino-9-benzylidene-9H-fluorene (*E*- or *Z*-) (2.68 g, 10 mmol) in DMF. The mixture was stirred at 40 °C until precipitation. The precipitated product was filtered and washed with a few drops of ethyl ether.

2.5. Spectral data of novel synthesized compounds

(Z)-1-(9-benzylidene-9H-fluoren-2-yl)-3-phenylurea (5a): Yield 79%, m.p. 272–273 °C; IR (ν , cm⁻¹): 3273 (NH), 3051 (C=C-H, Aromatic C-H), 1651 (C=O), 1551 (O=C-NH), 1222 (C-N); ¹H

NMR (300 MHz, DMSO- d_6 , ppm): 6.95 (t, 1H, $J = 7.3$ Hz, =CH), 7.23–7.92 (m, 17H, Ar-H), 8.53 (s, 1H, -NH), 8.57 (s, 1H, -NH); ^{13}C NMR (75 MHz, DMSO- d_6 , ppm): 92.6, 114.9, 118.8, 119.8, 120.0, 121.1, 121.3, 122.5, 127.0, 128.8, 129.1, 129.3, 129.5, 129.9, 135.7, 135.9, 136.6, 137.0, 139.2, 139.7, 140.3, 153.1; Anal. Calcd. for $\text{C}_{27}\text{H}_{20}\text{N}_2\text{O}$: C: 83.48; H: 5.19; N: 7.21. Found: C: 82.92; H: 5.70; N: 7.07.

(E)-1-(9-benzylidene-9H-fluoren-2-yl)-3-phenylurea (5b): Yield 82%, m.p. 277–278 °C; IR (ν , cm^{-1}): 3290 (NH), 3076 and 3024 (C=C-H, Aromatic C-H), 1637 (C=O), 1553 (O=C-NH), 1220 (C-N); ^1H NMR (300 MHz, DMSO- d_6 , ppm): 6.99–7.78 (m, 17H, =CH and Ar-H), 8.08 (s, 1H, Ar-H), 8.77 (s, 1H, -NH), 8.82 (s, 1H, -NH); ^{13}C NMR (75 MHz, DMSO- d_6 , ppm): 98.2, 111.9, 119.7, 119.8, 120.1, 120.9, 121.4, 122.7, 126.8, 129.0, 129.4, 129.5, 129.8, 129.9, 135.8, 136.2, 136.8, 137.2, 138.9, 139.3, 139.7, 140.4, 153.5; Anal. Calcd. for $\text{C}_{27}\text{H}_{20}\text{N}_2\text{O}$: C: 83.48; H: 5.19; N: 7.21. Found: C: 83.70; H: 5.62; N: 7.30.

(Z)-1-(9-benzylidene-9H-fluoren-2-yl)-3-(4-methylphenyl)urea (5c): Yield 97%, m.p. 262–263 °C; IR (ν , cm^{-1}): 3323 (NH), 3068 and 3022 (C=C-H, Aromatic C-H), 2924 (Aliphatic C-H), 1653 (C=O), 1556 (O=C-NH), 1236 (C-N); ^1H NMR (300 MHz, DMSO- d_6 , ppm): 2.22 (s, 3H, -CH $_3$), 7.05 (d, 2H, $J = 8.2$ Hz, =CH, Ar-H), 7.24–7.91 (m, 15H, Ar-H), 8.47 (s, 1H, -NH), 8.49 (s, 1H, -NH); ^{13}C NMR (75 MHz, DMSO- d_6 , ppm): 21.0, 114.7, 118.9, 119.8, 119.8, 121.1, 121.3, 126.9, 128.91, 129.1, 129.4, 129.6, 129.9, 129.9, 131.3, 135.5, 135.9, 136.7, 137.0, 137.8, 139.2, 139.4, 139.7, 153.1; Anal. Calcd. for $\text{C}_{28}\text{H}_{22}\text{N}_2\text{O}$: C: 83.56; H: 5.51; N: 6.96. Found: C: 82.84; H: 5.26; N: 6.32.

(E)-1-(9-benzylidene-9H-fluoren-2-yl)-3-(4-methylphenyl)urea (5d): Yield 95%, m.p. 282–283 °C; IR (ν , cm^{-1}): 3298 (NH), 3061 and 3026 (C=C-H, Aromatic C-H), 2910 (Aliphatic C-H), 1631 (C=O), 1553 (O=C-NH), 1228 (C-N); ^1H NMR (300 MHz, DMSO- d_6 , ppm): 2.23 (s, 3H, -CH $_3$), 7.00 (t, 1H, $J = 8.0$ Hz, =CH), 7.03–7.77 (m, 15H, Ar-H), 8.07 (s, 1H, Ar-H), 8.66 (s, 1H, -NH), 8.78 (s, 1H, -NH); ^{13}C NMR (75 MHz, DMSO- d_6 , ppm): 21.0, 111.2, 119.0, 119.5, 120.2, 120.9, 124.2, 126.6, 128.7, 129.3, 129.6, 129.8, 129.9, 129.9, 131.4, 133.3, 136.2, 136.3, 136.9, 137.8, 140.1, 140.3, 141.6, 153.4; Anal. Calcd. for $\text{C}_{28}\text{H}_{22}\text{N}_2\text{O}$: C: 83.56; H: 5.51; N: 6.96. Found: C: 82.97; H: 5.09; N: 6.70.

(Z)-1-(9-benzylidene-9H-fluoren-2-yl)-3-(3-methoxyphenyl)urea (5e): Yield 92%, m.p. 242–243 °C; IR (ν , cm^{-1}): 3305 (NH), 3049 and 3011 (C=C-H,

Aromatic C-H), 2833 (Aliphatic C-H), 1641 (C=O), 1552 (O=C-NH), 1220 (C-O-C); ^1H NMR (300 MHz, DMSO- d_6 , ppm): 3.74 (s, 3H, -OCH $_3$), 6.55 (d, 1H, $J = 8.2$ Hz, =CH), 6.90 (d, 1H, $J = 7.9$ Hz, Ar-H), 7.15–7.93 (m, 15H, Ar-H), 8.58 (s, 1H, -NH), 8.71 (s, 1H, -NH); ^{13}C NMR (75 MHz, DMSO- d_6 , ppm): 55.6, 104.5, 111.1, 114.9, 117.9, 119.8, 119.9, 121.1, 121.3, 126.9, 129.0, 129.4, 129.4, 129.9, 129.9, 130.2, 135.7, 135.9, 136.7, 137.1, 139.2, 139.2, 139.7, 141.5, 153.0, 160.3; Anal. Calcd. for $\text{C}_{28}\text{H}_{22}\text{N}_2\text{O}_2$: C: 80.36; H: 5.30; N: 6.69. Found: C: 80.09; H: 5.13; N: 6.21.

(E)-1-(9-benzylidene-9H-fluoren-2-yl)-3-(3-methoxyphenyl)urea (5f): Yield 90%, m.p. 241–242 °C; IR (ν , cm^{-1}): 3273 (NH), 3068 and 3021 (C=C-H, Aromatic C-H), 2864 (Aliphatic C-H), 1633 (C=O), 1554 (O=C-NH), 1157 (C-O-C); ^1H NMR (300 MHz, DMSO- d_6 , ppm): 3.73 (s, 3H, -OCH $_3$), 6.55 (d, 1H, $J = 7.9$ Hz, =CH), 6.98–7.79 (m, 15H, Ar-H), 8.08 (s, 1H, Ar-H), 8.77 (s, 1H, -NH), 8.80 (s, 1H, -NH); ^{13}C NMR (75 MHz, DMSO- d_6 , ppm): 55.6, 104.7, 108.0, 111.3, 111.4, 119.7, 120.2, 120.9, 124.2, 126.6, 128.7, 129.0, 129.3, 129.6, 129.6, 129.8, 130.3, 133.4, 136.3, 136.4, 136.9, 139.9, 140.4, 141.6, 153.3, 160.4; Anal. Calcd. for $\text{C}_{28}\text{H}_{22}\text{N}_2\text{O}_2$: C: 80.36; H: 5.30; N: 6.69. Found: C: 80.05; H: 5.17; N: 6.32.

(Z)-1-(9-benzylidene-9H-fluoren-2-yl)-3-(4-flourophenyl)urea (5g): Yield 85%, m.p. 267–268 °C; IR (ν , cm^{-1}): 3261 (NH), 3053 and 3016 (C=C-H, Aromatic C-H), 1649 (C=O), 1546 (O=C-NH), 1217 (C-N); ^1H NMR (300 MHz, DMSO- d_6 , ppm): 7.13 (t, 1H, $J = 8.9$ Hz, =CH), 7.26–7.93 (m, 16H, Ar-H), 8.57 (s, 1H, -NH), 8.64 (s, 1H, -NH); ^{13}C NMR (75 MHz, DMSO- d_6 , ppm): 114.9, 115.8, 116.1, 119.8, 119.9, 120.5, 120.6, 121.1, 121.3, 126.9, 129.0, 129.4, 129.9, 135.7, 135.9, 136.7, 137.0, 139.2, 139.3, 139.7, 153.1, 156.4, 159.5; Anal. Calcd. for $\text{C}_{27}\text{H}_{19}\text{FN}_2\text{O}$: C: 79.79; H: 4.71; N: 6.89. Found: C: 79.14; H: 4.53; N: 6.95.

(E)-1-(9-benzylidene-9H-fluoren-2-yl)-3-(4-flourophenyl)urea (5h): Yield 78%, m.p. 279–280 °C; IR (ν , cm^{-1}): 3280 (NH), 3051 and 3018 (C=C-H, Aromatic C-H), 1633 (C=O), 1557 (O=C-NH), 1211 (C-N); ^1H NMR (300 MHz, DMSO- d_6 , ppm): 7.08 (t, 1H, $J = 8.0$ Hz, =CH), 7.16–7.81 (m, 15H, Ar-H), 8.12 (s, 1H, Ar-H), 8.84 (s, 1H, -NH), 8.85 (s, 1H, -NH); ^{13}C NMR (75 MHz, DMSO- d_6 , ppm): 111.4, 115.8, 116.1, 119.6, 120.2, 120.7, 120.8, 120.9, 124.2, 128.7, 129.0, 129.3, 129.6, 129.8, 133.4, 136.3, 136.3, 136.7, 136.9, 139.9, 140.4, 141.6, 153.4; Anal. Calcd. for $\text{C}_{27}\text{H}_{19}\text{FN}_2\text{O}$: C: 79.79; H: 4.71; N: 6.89. Found: C: 79.21; H: 4.51; N: 6.92.

(Z)-1-(9-benzylidene-9H-fluoren-2-yl)-3-(4-chlorophenyl)urea (5i): Yield 72%, m.p. 244–245 °C; IR (ν , cm^{-1}): 3280 (NH), 3049 and 3019 (C=C-H, Aromatic C-H), 1639 (C=O), 1547 (O=C-NH), 1224 (C-N); ^1H NMR (300 MHz, DMSO- d_6 , ppm): 7.27–7.92 (m, 17H, =CH and Ar-H), 8.58 (s, 1H, -NH), 8.71 (s, 1H, -NH); ^{13}C NMR (75 MHz, DMSO- d_6 , ppm): 115.1, 119.8, 120.1, 120.4, 120.4, 121.1, 121.3, 126.0, 127.0, 129.1, 129.3, 129.4, 129.9, 130.0, 135.8, 135.9, 136.6, 137.0, 139.1, 139.2, 139.3, 139.7, 153.0; Anal. Calcd. for $\text{C}_{27}\text{H}_{19}\text{ClN}_2\text{O}$: C: 76.68; H: 4.53; N: 6.62. Found: C: 75.81; H: 4.02; N: 6.38.

(E)-1-(9-benzylidene-9H-fluoren-2-yl)-3-(4-chlorophenyl)urea (5j): Yield 75%, m.p. 259–260 °C; IR (ν , cm^{-1}): 3292 (NH), 3057 and 3022 (C=C-H, Aromatic C-H), 1637 (C=O), 1548 (O=C-NH), 1224 (C-N); ^1H NMR (300 MHz, DMSO- d_6 , ppm): 7.05 (t, 1H, $J = 7.6$ Hz, =CH), 7.30–7.78 (m, 15H, Ar-H), 8.55 (s, 1H, Ar-H), 8.86 (s, 1H, -NH), 8.92 (s, 1H, -NH); ^{13}C NMR (75 MHz, DMSO- d_6 , ppm): 111.5, 119.8, 120.2, 120.4, 120.5, 120.9, 124.3, 126.1, 128.7, 129.0, 129.3, 129.4, 129.6, 129.8, 133.5, 136.3, 136.4, 136.9, 139.4, 139.7, 140.4, 141.5, 153.3; Anal. Calcd. for $\text{C}_{27}\text{H}_{19}\text{ClN}_2\text{O}$: C: 76.68; H: 4.53; N: 6.62. Found: C: 75.95; H: 4.17; N: 6.34.

(Z)-1-(9-benzylidene-9H-fluoren-2-yl)-3-(3-chlorophenyl)urea (5k): Yield 72%, m.p. 222–223 °C; IR (ν , cm^{-1}): 3261 (NH), 3068 and 3034 (C=C-H, Aromatic C-H), 1643 (C=O), 1548 (O=C-NH), 1213 (C-N); ^1H NMR (300 MHz, DMSO- d_6 , ppm): 6.99 (d, 1H, $J = 8.0$ Hz, =CH), 7.21–7.90 (m, 16H, Ar-H), 8.62 (s, 1H, -NH), 8.78 (s, 1H, -NH); ^{13}C NMR (75 MHz, DMSO- d_6 , ppm): 115.2, 117.3, 118.2, 119.8, 120.2, 121.1, 121.3, 122.1, 127.0, 128.9, 129.0, 129.4, 129.7, 129.9, 131.1, 133.9, 135.9, 136.1, 136.7, 137.1, 138.9, 139.1, 139.7, 141.9, 152.9; Anal. Calcd. for $\text{C}_{27}\text{H}_{19}\text{ClN}_2\text{O}$: C: 76.68; H: 4.53; N: 6.62. Found: C: 75.92; H: 4.37; N: 6.20.

(E)-1-(9-benzylidene-9H-fluoren-2-yl)-3-(3-chlorophenyl)urea (5l): Yield 73%, m.p. 240–241 °C; IR (ν , cm^{-1}): 3271 (NH), 3072 and 3020 (C=C-H, Aromatic C-H), 1635 (C=O), 1551 (O=C-NH), 1220 (C-N); ^1H NMR (300 MHz, DMSO- d_6 , ppm): 7.01 (t, 1H, $J = 7.8$ Hz, =CH), 7.26–7.78 (m, 15H, Ar-H), 8.09 (s, 1H, Ar-H), 8.88 (s, 1H, -NH), 8.97 (s, 1H, -NH); ^{13}C NMR (75 MHz, DMSO- d_6 , ppm): 111.6, 117.4, 118.3, 119.9, 120.2, 120.9, 122.2, 124.3, 126.7, 128.7, 129.0, 129.3, 129.6, 129.8, 131.1, 133.6, 133.9, 136.2, 136.4, 136.9, 139.6, 140.4, 141.5, 141.9, 153.2; Anal. Calcd. for $\text{C}_{27}\text{H}_{19}\text{ClN}_2\text{O}$: C: 76.68; H: 4.53; N: 6.62. Found: C: 76.08; H: 4.65; N: 6.26.

(Z)-1-(9-benzylidene-9H-fluoren-2-yl)-3-(3,4-dichlorophenyl)urea (5m): Yield 79%, m.p. 248–249 °C; IR (ν , cm^{-1}): 3288 (NH), 3059 and 3026 (C=C-H, Aromatic C-H), 1633 (C=O), 1548 (O=C-NH), 1228 (C-N); ^1H NMR (300 MHz, DMSO- d_6 , ppm): 7.30–7.93 (m, 16H, =CH and Ar-H), 8.71 (s, 1H, -NH), 8.92 (s, 1H, -NH); ^{13}C NMR (75 MHz, DMSO- d_6 , ppm): 115.3, 119.0, 119.8, 120.0, 120.3, 121.0, 121.3, 123.8, 127.1, 128.9, 129.0, 129.4, 129.6, 129.9, 131.2, 131.7, 135.9, 136.1, 136.6, 137.1, 138.8, 139.1, 139.7, 140.6, 152.9; Anal. Calcd. for $\text{C}_{27}\text{H}_{18}\text{Cl}_2\text{N}_2\text{O}$: C: 70.91; H: 3.97; N: 6.13. Found: C: 69.95; H: 3.39; N: 5.87.

(E)-1-(9-benzylidene-9H-fluoren-2-yl)-3-(3,4-dichlorophenyl)urea (5n): Yield 78%, m.p. 273–274 °C; IR (ν , cm^{-1}): 3278 (NH), 3074 and 3024 (C=C-H, Aromatic C-H), 1639 (C=O), 1547 (O=C-NH), 1222 (C-N); ^1H NMR (300 MHz, DMSO- d_6 , ppm): 7.01 (t, 1H, $J = 7.9$ Hz, =C), 7.26–7.78 (m, 13H, Ar-H), 7.92 (s, 1H, Ar-H), 8.08 (s, 1H, Ar-H), 8.93 (s, 1H, -NH), 9.06 (s, 1H, -NH); ^{13}C NMR (75 MHz, DMSO- d_6 , ppm): 111.7, 119.1, 119.9, 120.0, 120.2, 120.9, 123.9, 124.2, 126.7, 128.8, 129.0, 129.3, 129.6, 129.8, 131.2, 131.8, 133.7, 136.2, 136.4, 136.9, 139.4, 140.4, 140.6, 141.5, 153.1; Anal. Calcd. for $\text{C}_{27}\text{H}_{18}\text{Cl}_2\text{N}_2\text{O}$: C: 70.91; H: 3.97; N: 6.13. Found: C: 70.13; H: 3.46; N: 5.95.

(Z)-1-(9-benzylidene-9H-fluoren-2-yl)-3-(4-nitrophenyl)urea (5o): Yield 91%, m.p. 287–288 °C; IR (ν , cm^{-1}): 3329 (NH), 3055 and 3022 (C=C-H, Aromatic C-H), 1666 (C=O), 1549 (O=C-NH), 1496 (O-N-O), 1240 (C-N); ^1H NMR (300 MHz, DMSO- d_6 , ppm): 7.26–7.93 (m, 15H, Ar-H), 8.17 (d, 2H, $J = 7.6$ Hz Ar-H), 8.83 (s, 1H, -NH), 9.35 (s, 1H, -NH); ^{13}C NMR (75 MHz, DMSO- d_6 , ppm): 115.3, 118.1, 119.8, 119.9, 120.3, 121.3, 125.9, 127.1, 129.1, 129.3, 129.4, 129.9, 129.9, 135.8, 136.2, 136.6, 137.0, 138.6, 139.0, 139.7, 141.6, 147.0, 152.5; Anal. Calcd. for $\text{C}_{27}\text{H}_{19}\text{N}_3\text{O}_3$: C: 74.81; H: 4.42; N: 9.69. Found: C: 74.59; H: 4.05; N: 10.06.

(E)-1-(9-benzylidene-9H-fluoren-2-yl)-3-(4-nitrophenyl)urea (5p): Yield 90%, m.p. 282–283 °C; IR (ν , cm^{-1}): 3284 (NH), 3057 and 3028 (C=C-H, Aromatic C-H), 1670 (C=O), 1548 (O=C-NH), 1495 (O-N-O), 1228 (C-N); ^1H NMR (300 MHz, DMSO- d_6 , ppm): 7.08 (t, 1H, $J = 7.9$ Hz, =CH₂), 7.22–7.83 (m, 14H, Ar-H), 8.21 (d, 2H, $J = 8.0$ Hz, Ar-H), 9.11 (s, 1H, -NH), 9.58 (s, 1H, -NH); ^{13}C NMR (75 MHz, DMSO- d_6 , ppm): 111.8, 118.2, 120.0, 120.3, 121.0, 124.3, 125.9, 126.8, 129.1, 129.3, 129.6, 129.8, 129.9, 134.0, 136.2, 136.4, 136.9, 139.2, 140.4, 141.4, 141.7, 147.1, 152.8; Anal. Calcd. for $\text{C}_{27}\text{H}_{19}\text{N}_3\text{O}_3$: C: 74.81; H: 4.42; N: 9.69. Found: C: 74.50; H: 4.13; N: 9.98.

(Z)-1-(9-benzylidene-9H-fluoren-2-yl)-3-phenylthiourea (5q): Yield 92%, m.p. 148–149 °C; IR (ν , cm^{-1}): 3217 (NH), 3051 and 3018 (C=C-H, Aromatic C-H), 1539 (S=C-N-H), 1255 (C-N); ^1H NMR (300 MHz, DMSO- d_6 , ppm): 7.25 (t, 1H, J = 7.9 Hz, =CH), 7.43–7.94 (m, 17H, Ar-H), 9.73 (s, 1H, -NH), 9.82 (s, 1H, -NH); ^{13}C NMR (75 MHz, DMSO- d_6 , ppm): 120.2, 120.4, 120.8, 121.4, 124.4, 124.6, 125.1, 127.5, 129.0, 129.4, 129.8, 130.0, 135.6, 136.5, 137.9, 138.8, 140.0, 140.2, 180.0; Anal. Calcd. for $\text{C}_{27}\text{H}_{20}\text{N}_2\text{S}$: C: 80.17; H: 4.98; N: 6.92; S: 7.93. Found: C: 80.31; H: 4.66; N: 6.57; S: 8.09.

(E)-1-(9-benzylidene-9H-fluoren-2-yl)-3-phenylthiourea (5r): Yield 98%, m.p. 151–152 °C; IR (ν , cm^{-1}): 3221 (NH), 3039 and 3019 (C=C-H, Aromatic C-H), 1524 (S=C-N-H), 1253 (C-N); ^1H NMR (300 MHz, DMSO- d_6 , ppm): 7.07–7.18 (m, 2H, =CH and Ar-H), 7.34–7.85 (m, 14H, Ar-H), 8.03 (s, 1H, Ar-H), 9.85 (s, 1H, -NH), 9.88 (s, 1H, -NH); ^{13}C NMR (75 MHz, DMSO- d_6 , ppm): 117.8, 120.2, 120.50, 120.7, 124.3, 124.4, 124.6, 125.2, 125.7, 127.2, 129.2, 129.7, 135.9, 136.0, 136.6, 136.8, 138.7, 139.4, 139.9, 140.1, 141.2, 180.0, 180.6; Anal. Calcd. for $\text{C}_{27}\text{H}_{20}\text{N}_2\text{S}$: C: 80.17; H: 4.98; N: 6.92; S: 7.93. Found: C: 80.28; H: 4.39; N: 6.40; S: 7.98.

(Z)-1-(9-benzylidene-9H-fluoren-2-yl)-3-(4-methylphenyl)thiourea (5s): Yield 95%, m.p. 116–117 °C; IR (ν , cm^{-1}): 3167 (NH), 3049 and 3024 (C=C-H, Aromatic C-H), 2920 (Aliphatic C-H), 1516 (S=C-NH), 1255 (C-N); ^1H NMR (300 MHz, DMSO- d_6 , ppm): 2.29 (s, 3H, -CH₃), 7.15 (t, 1H, J = 8.2 Hz, =CH), 7.28–7.97 (m, 16H, Ar-H), 9.64 (s, 1H, -NH), 9.73 (s, 1H, -NH); ^{13}C NMR (75 MHz, DMSO- d_6 , ppm): 21.2, 120.2, 120.5, 120.8, 121.4, 124.7, 124.9, 125.2, 127.5, 129.1, 129.4, 129.5, 129.7, 129.8, 130.0, 134.4, 135.6, 136.5, 137.5, 137.9, 138.8, 138.8, 139.9, 180.1; Anal. Calcd. for $\text{C}_{28}\text{H}_{22}\text{N}_2\text{S}$: C: 80.35; H: 5.30; N: 6.69; S: 7.66. Found: C: 80.02; H: 5.14; N: 6.31; S: 7.74.

(E)-1-(9-benzylidene-9H-fluoren-2-yl)-3-(4-methylphenyl)thiourea (5t): Yield 94%, m.p. 116–117 °C; IR (ν , cm^{-1}): 3207 (NH), 3024 and 3024 (C=C-H, Aromatic C-H), 2918 (Aliphatic C-H), 1518 (S=C-NH), 1253 (C-N); ^1H NMR (300 MHz, DMSO- d_6 , ppm): 2.27 (s, 3H, -CH₃), 7.08 (t, 1H, J = 8.0 Hz, =CH), 7.14–7.72 (m, 15H, Ar-H), 8.02 (s, 1H, Ar-H), 9.79 (s, 1H, -NH), 9.95 (s, 1H, -NH); ^{13}C NMR (75 MHz, DMSO- d_6 , ppm): 21.2, 119.7, 120.1, 120.6, 121.8, 125.0, 125.4, 126.9, 128.9, 129.2, 129.6, 129.7, 130.0, 130.2, 133.9, 135.6, 136.7, 138.0, 138.7, 139.4, 139.5, 139.8, 140.2, 180.8; Anal. Calcd. for $\text{C}_{28}\text{H}_{22}\text{N}_2\text{S}$: C:

80.35; H: 5.30; N: 6.69; S: 7.66. Found: C: 80.19; H: 5.11; N: 6.24; S: 7.71.

(Z)-1-(9-benzylidene-9H-fluoren-2-yl)-3-(4-methoxyphenyl)thiourea (5u): Yield 89%, m.p. 136–137 °C; IR (ν , cm^{-1}): 3157 (NH), 3049 (C=C-H, Aromatic C-H), 2955 (Aliphatic C-H), 1510 (S=C-NH), 1240 (Ar-O-CH₃); ^1H NMR (300 MHz, DMSO- d_6 , ppm): 3.75 (s, 3H, -OCH₃), 6.92 (d, 2H, J = 8.4 Hz, Ar-H), 7.28–7.96 (m, 15H, =CH and Ar-H), 9.57 (s, 1H, -NH), 9.68 (s, 1H, -NH); ^{13}C NMR (75 MHz, DMSO- d_6 , ppm): 55.9, 114.3, 120.2, 120.4, 120.7, 121.4, 125.2, 126.7, 127.5, 129.0, 129.1, 129.4, 129.8, 130.0, 132.9, 135.7, 136.5, 137.9, 138.8, 138.9, 140.0, 157.2, 180.3; Anal. Calcd. for $\text{C}_{28}\text{H}_{22}\text{N}_2\text{OS}$: C: 77.39; H: 5.10; N: 6.45; S: 7.38. Found: C: 77.53; H: 4.92; N: 6.20; S: 7.47.

(E)-1-(9-benzylidene-9H-fluoren-2-yl)-3-(4-methoxyphenyl)thiourea (5v): Yield 90%, m.p. 147–148 °C; IR (ν , cm^{-1}): 3159 (NH), 3048 (C=C-H, Aromatic C-H), 2911 (Aliphatic C-H), 1511 (S=C-NH), 1240 (Ar-O-CH₃); ^1H NMR (300 MHz, DMSO- d_6 , ppm): 3.73 (s, 3H, -OCH₃), 6.93 (d, 2H, J = 8.2 Hz, Ar-H), 7.18 (t, 1H, J = 7.9 Hz, =CH), 7.32–7.90 (m, 13H, Ar-H), 8.05 (s, 1H, Ar-H), 9.69 (s, 1H, -NH), 9.74 (s, 1H, -NH); ^{13}C NMR (75 MHz, DMSO- d_6 , ppm): 55.9, 114.4, 115.8, 117.8, 120.5, 120.7, 124.3, 125.6, 126.6, 126.9, 128.0, 129.1, 129.4, 129.8, 132.9, 135.8, 136.1, 136.6, 136.9, 139.6, 139.9, 141.3, 157.3, 180.9; Anal. Calcd. For $\text{C}_{28}\text{H}_{22}\text{N}_2\text{OS}$: C: 77.39; H: 5.10; N: 6.45; S: 7.38. Found: C: 77.65; H: 4.87; N: 6.18; S: 7.49.

3. CA ENZYME ASSAY

3.1. Preparation and purification of haemolysate from red blood cells

Blood samples (25 ml) were taken from healthy human volunteers. They were anticoagulated with acid-citrate-dextrose, centrifuged at 5000 rpm for 20 min at 4 °C and the supernatant was removed. The packed erythrocytes were washed three times with 0.9 % NaCl and then haemolysed in cold water. The ghosts and any intact cells were removed by centrifugation at 15000 rpm for 25 min at 4 °C, and the pH of the haemolysate was adjusted to pH 8.5 with solid Tris-base. The haemolysate (25 ml) was applied to an affinity column containing -sulfonamide- L-tyrosine -Sepharose-4B [38] equilibrated with 25 mM Tris-HCl/0.1 M Na₂SO₄ (pH 8.5). The affinity gel was washed with 50 ml of 25 mM Tris-HCl/22 mM Na₂SO₄ (pH 8.5). The human CA (hCA)

isozymes were then eluted with 0.1 M NaCl/25 mM Na₂HPO₄ (pH 6.3) and 0.1 M CH₃COONa/0.5 M NaClO₄ (pH 5.6), which recovered hCA I and II respectively. Fractions of 3 ml were collected and their absorbance measured at 280 nm.

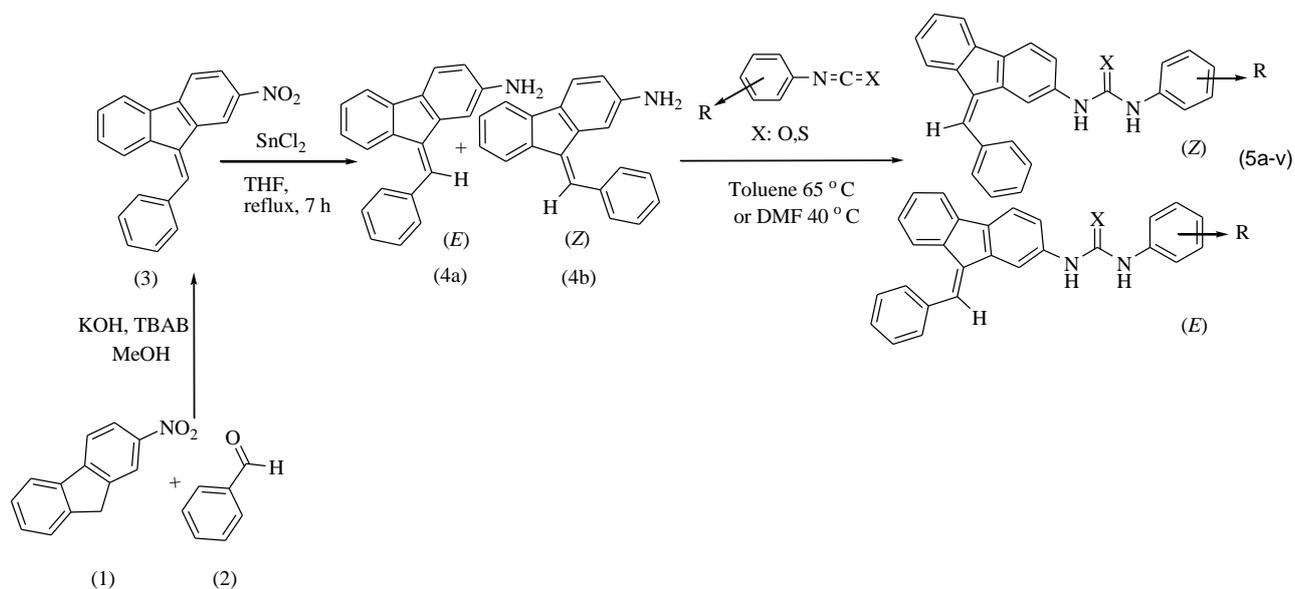
3.2. *In vitro* inhibition studies

CA activity was measured by the Maren method, which is based on determination of the time required for the pH to decrease from 10.0 to 7.4 due to CO₂ hydration [39]. The assay solution was 0.5 M Na₂CO₃/0.1 M NaHCO₃ (pH 10.0) and phenol red was added as the pH indicator. CO₂-hydratase activity (enzyme units (EU)) was calculated by using the equation $(t_0 - t_c)/t_c$ where t_0 and t_c are the times for pH change of the non-enzymatic and the enzymatic reactions, respectively.

For the inhibition studies of synthesized compounds, different concentrations of these compounds were added to the enzyme. Activity percentage values of CA for each concentration of each compound were determined by regression analysis using Microsoft Office 2000 Excel. CA enzyme activity without urea solution was deemed to be 100%.

4. RESULTS AND DISCUSSION

2-Nitro-9-benzylidene-9H-fluorene (**3**) was synthesized from 2-nitrofluorene (**1**) and the compound was reduced with tin (II) chloride in THF. The *E*- and *Z*-isomers of 2-nitro-9-benzylidene-9H-fluorene (**4a–b**) were reacted with isocyanates/isothiocyanates to get the final products (**5a–v**) at high yields. The synthetic procedures are depicted in Scheme 1.



	5a	5b	5c	5d	5e	5f	5g	5h
X	O	O	O	O	O	O	O	O
R	H	H	4-CH ₃	4-CH ₃	3-OMe	3-OMe	4-F	4-F
Conf.	<i>Z</i> -	<i>E</i> -	<i>Z</i> -	<i>E</i> -	<i>Z</i> -	<i>E</i> -	<i>Z</i> -	<i>E</i> -
	5i	5j	5k	5l	5m	5n	5o	5p
X	O	O	O	O	O	O	O	O
R	4-Cl	4-Cl	3-Cl	3-Cl	3,4-di-Cl	3,4-di-Cl	4-NO ₂	4-NO ₂
Conf.	<i>Z</i> -	<i>E</i> -	<i>Z</i> -	<i>E</i> -	<i>Z</i> -	<i>E</i> -	<i>Z</i> -	<i>E</i> -
	5q	5r	5s	5t	5u	5v		
X	S	S	S	S	S	S		
R	H	H	4-CH ₃	4-CH ₃	4-OMe	4-OMe		
Conf.	<i>Z</i> -	<i>E</i> -	<i>Z</i> -	<i>E</i> -	<i>Z</i> -	<i>E</i> -		

Scheme 1. Synthesis of (*E* or *Z*)-1-(9-benzylidene-9H-fluoren-2-yl)-3-phenylurea/thiourea derivatives

The synthesized compounds were characterized by ^1H NMR, ^{13}C NMR, IR and elemental analysis. From the ^1H NMR spectra, the resonance due to the hydrogen attached to the amide nitrogen was between 8.50 and 10.00 ppm. The signals for aromatic and vinylic protons were between 7.00 and 8.50 ppm. From the ^{13}C NMR spectra, carbon atoms of urea carbonyl were observed between 182 and 150 ppm. In the infrared spectra of compounds **5a–v**, it was possible to observe the absorptions between 3250 and 3450 cm^{-1} relating to N-H stretching and absorptions at 1650–1750 cm^{-1} from the urea carbonyl moiety stretching. Furthermore, absorptions between 1180 and 1280 cm^{-1} indicated C-N stretching.

To evaluate the hCA I and II inhibitory effects, all compounds were subjected to hCA I and II inhibition assays with CO_2 as a substrate. The results showed that these compounds (**5a–v**) inhibited the CA enzyme activity. The IC_{50} values of **5a–v** analogues for hCA I and II are summarized in Table 1. The IC_{50} values were between 21.4 and 211.4 μM for hCA I enzyme activity and between 25.3 and 82.4 μM for hCA II. Among the compounds, **5f** ($\text{IC}_{50} = 21.4 \mu\text{M}$) was found to be the most active compound for hCA I inhibitory activity and **5s** ($\text{IC}_{50} = 25.3 \mu\text{M}$) showed the highest hCA II inhibitory activity.

Table 1

The IC_{50} values of (*E* or *Z*)-1-(9-benzylidene-9H-fluorene-2-yl)-3-phenylurea/thiourea derivatives

Comp./ Conf.	X	R	hCA I (μM)	hCA II (μM)	Comp./ Conf.	X	R	hCA I (μM)	hCA II (μM)
5a/ <i>Z</i>	O	H	43.9	56.9	5l/ <i>E</i>	O	3-Cl	52.7	63.8
5b/ <i>E</i>	O	H	32.75	64.98	5m/ <i>Z</i>	O	3,4-di-Cl	67.0	38.3
5c/ <i>Z</i>	O	4- CH_3	66.9	29.2	5n/ <i>E</i>	O	3,4-di-Cl	72.9	51.5
5d/ <i>E</i>	O	4- CH_3	74.6	25.6	5o/ <i>Z</i>	O	4- NO_2	23.1	35.9
5e/ <i>Z</i>	O	3- OCH_3	32.0	40.55	5p/ <i>E</i>	O	4- NO_2	37.4	28.6
5f/ <i>E</i>	O	3- OCH_3	21.4	62.8	5q/ <i>Z</i>	S	H	157.3	29.2
5g/ <i>Z</i>	O	4-F	73.5	63.0	5r/ <i>E</i>	S	H	58.0	41.4
5h/ <i>E</i>	O	4-F	50.44	51.3	5s/ <i>Z</i>	S	4- CH_3	35.01	25.3
5i/ <i>Z</i>	O	4-Cl	66.5	36.8	5t/ <i>E</i>	S	4- CH_3	62.7	43.9
5j/ <i>E</i>	O	4-Cl	211.4	55.1	5u/ <i>Z</i>	S	4- OCH_3	24.6	68.6
5k/ <i>Z</i>	O	3-Cl	64.8	37.9	5v/ <i>E</i>	S	4- OCH_3	23.0	82.4

The following conclusions should be noted regarding the CA inhibitory data of Table 1.

(i) The slow cytosolic isoform hCA I was inhibited by the 9-benzylidene-9H-fluorene-substituted diaryl urea and thiourea derivatives with inhibition constants in the range 21.4–211.4 μM . The best hCA I inhibitor among the novel compounds was **5f**. *E*-isomers of urea and thiourea compounds, which do not have any group at the phenyl ring, showed a higher inhibitory effect than *Z*-isomers for hCA I. A methoxy group at the phenyl ring had a greater inhibitory effect for hCA I than for hCA II, while a methyl group at the phenyl ring had a higher inhibitory effect for hCA II than for hCA I.

(ii) The second off-target isoform (hCA II), which is in fact the physiologically dominant cytosolic isozyme, was also inhibited by all the compounds with inhibition constants in the range 25.3–82.4 μM . The best hCA II inhibitor among the novel compounds was **5s**. *Z*-isomers of the synthesized compounds were generally more effective in

the inhibition of hCA II. Both *Z*- and *E*-isomers of the synthesized compounds containing $-\text{NO}_2$ groups at the phenyl ring had a higher inhibitory effect. A chlorine atom at the *meta*-position of the phenyl ring exhibited a greater inhibitory effect than at the *para*-position.

In conclusion, we have evaluated the effect of urea (**5a–p**) and thiourea (**5q–v**) derivatives on hCA I and II purified from human erythrocytes and structure–activity relationships were examined. The synthesized compounds inhibited the hCA I and II isoenzyme activities. The urea derivatives as inhibitor were bound within the enzyme active site [19, 40]. We assume that the synthesized fluorene-containing urea/thiourea derivatives inhibited hCA I and II in the same way or that the fluorenyl moiety interacted with the hydrophobic pocket of the enzyme.

In summary, enzyme inhibition is an important issue for drug design and biochemical applications [41–45]. Our results suggest that these novel compounds are likely to be adopted as candidates

for the treatment of glaucoma and that they should be further evaluated in *in vivo* studies.

Acknowledgements: This work was supported by the Research Fund of the Sakarya University. Project Number: 2012-02-04-043.

REFERENCES

- [1] M. S. Novikov, A. F. Khlebnikov, M. A. Egarmin, M. V. Shevchenko, V. A. Khlebnikov, R. R. Kostikov, D. Vidovic, Regioselectivity of the 1,3-Dipolar Cycloaddition of Fluorinated Fluoren-9-iminium Ylides to Heteroelement-Containing Dipolarophiles: Experimental and Quantum-Chemical Study, *Russian J. Org. Chem.*, **42**, 1800–1812 (2006).
- [2] V. Lukes, D. Vegh, P. Hrdlovic, M. Stefko, K. Matuszna, V. Laurinc, Synthesis, theoretical characterisation and spectra of thiophene-fluorene π -conjugated derivatives, *Synthetic Metals*, **148**, 179–186 (2005).
- [3] S. L. Tao, Z. K. Peng, X. H. Zhang, P. F. Wang, C. S. Lee, S. T. Lee, Highly Efficient Non-Doped Blue Organic Light-Emitting Diodes Based on Fluorene Derivatives with High Thermal Stability, *Advanced Functional Mat.*, **15**, 1716–1721 (2005).
- [4] G. Hsu, J. R. Kiefer, D. Burnouf, O. J. Becherel, R. P. P. Fuchs, L. S. Beese, Observing Translesion Synthesis of an Aromatic Amine DNA Adduct by a High-fidelity DNA Polymerase, *J. Biol. Chem.*, **279**, 50280–50285 (2004).
- [5] S. Schulman, Fluorene Derivatives for Cancer Research, *J. Org. Chem.*, **14**, 382–387 (1949).
- [6] L. A. Pinck, On the Carcinogenesis of 2-Substituted Fluorenes, *Comments and Communications*, **109**, 209 (1949).
- [7] PATENT: S. Ahmed, P. C. Gambacorti, P. G. Goekjian, D. Gueyrard, R. H. Gunby, F. Popowycz, L. Scapozza, C. Schneider, A. Zambon, US20110112110 A1; (2011).
- [8] P. Marinova, M. Marinov, Y. Feodorova, M. Kazakova, D. Georgiev, E. Trendafilova, P. Penchev, V. Sarafian, N. Stoyanov, Synthesis, antimicrobial and *in vitro* anti-proliferative activity of 4'-bromo-(9'-fluorene)-spiro-5-(2,4-dithiohydantoin) against tumor cells, *Scientific Works: University of Ruse "Angel Kanchev"* **52**, 33–37 (2013).
- [9] B. Beije, L. Möller, 2-nitrofluorene and related compounds: prevalence and biological effects, *Mutation Research/Reviews in Genetic Toxicology*, **196**, 177–209 (1988).
- [10] C. Jing, L. Yang, F. Junxiang, 9-Benzylidene-9H-fluorene Derivatives Linked to Monoaza-15-crown-5: Synthesis and Metal Ion Sensing, *Chin. J. Chem.*, **30**, 1571–1574 (2012).
- [11] M. Houimel, J-P. Mach, I. Corthésy-Theulaz, B. Corthésy, I. Fisch, New inhibitors of Helicobacter pylori urease holoenzyme selected from phage-displayed peptide libraries, *Eur. J. Biochem.*, **262**, 774–780 (1999).
- [12] I. J. M. Rosenstein, J. M. T. Hamilton-Miller, D. M. Musher, Inhibitors of urease as chemotherapeutic agents, *Crit. Rev. Microbiol.*, **11**, 1–12 (1984).
- [13] H. Dulude, R. Salvador, G. Gallant, Synthesis and anti-HIV activity of new urea and nitrosourea derivatives of diamino acids, *Bioorg. Med. Chem.*, **3**, 151–160 (1995).
- [14] N. Gencer, D. Demir, F. Sonmez, M. Kucukislamoglu, New saccharin derivatives as tyrosinase inhibitors, *Bioorg. Med. Chem.*, **20**, 2811–2821 (2012).
- [15] A. R. Nixha, M. Arslan, Y. Atalay, N. Gencer, A. Ergun, O. Arslan, Synthesis and theoretical calculations of carbazole substituted chalcone urea derivatives and studies their polyphenol oxidase enzyme activity, *J. Enzyme Inhib. Med. Chem.*, **28**, 808–815 (2013).
- [16] G. Madhava, K. Venkata Subbaiah, R. Sreenivasulu, C. Naga Raju, Synthesis of novel urea and thiourea derivatives of diphenylphosphoramidate and their antimicrobial activity *Der Pharmacia Lettre*, **4**, 1194–1201 (2012).
- [17] M. Avalos, R. Babiano, P. Cintas, M. M. Chavero, F. J. Higes, J. L. Jimenez, J. C. Palacios, G. Silvero, Reactions of 2-amino-2-thiazolines with isocyanates and isothiocyanates. Chemical and computational studies on the regioselectivity, adduct rearrangement, and mechanistic pathways, *J. Org. Chem.*, **65**, 8882–8892 (2000).
- [18] M. D'hooghe, N. De Kimpe, Synthetic approaches towards 2-iminothiazolidines: an overview, *Tetrahedron*, **62**, 513–535 (2006).
- [19] F. Pacchiano, F. Carta, P. C. McDonald, Y. Lou, D. Vullo, A. Scozzafava, S. Dedhar, C. T. Supuran, Ureido-substituted benzenesulfonamides potently inhibit carbonic anhydrase IX and show antimetastatic activity in a model of breast cancer metastasis, *J. Med. Chem.*, **54**, 1896–1902 (2011).
- [20] C. T. Supuran, Carbonic anhydrases: novel therapeutic applications for inhibitors and activators, *Nat. Rev. Drug Disc.*, **7**, 168–181 (2008).
- [21] A. Thiry, J. M. Dogné, B. Masereel, C. T. Supuran, Targeting tumor associated carbonic anhydrase IX in cancertherapy, *Trends Pharmacol. Sci.*, **27**, 566–573 (2006).
- [22] J. M. McKiernan, R. Buttyan, N. H. Bander, M. D. Stifelman, A. E. Katz, M. W. Chen, C. A. Olsson, I. S. Sawczuk, Expression of the tumor-associated gene MN: A potential biomarker for human renal cell carcinoma, *Cancer Res.*, **57**, 2362–2365 (1997).
- [23] A. Scozzafava, A. Mastrolorenzo, C.T. Supuran, Carbonic anhydrase inhibitors and activators and their use in therapy, *Expert Opin. Ther. Pat.*, **16**, 1627–1664 (2006).
- [24] C. T. Supuran, A. Scozzafava, A. Casini, Carbonic anhydrase inhibitors, *Med. Res. Rev.*, **23**, 146–189 (2003).
- [25] K. S. Smith, J. G. Ferry, Prokaryotic carbonic anhydrases, *FEMS Microbiol. Rev.*, **24**, 335–366 (2000).
- [26] T. Stams, D. W. Christianson, *The Carbonic Anhydrases: New Horizons*, Birkhauser Verlag, Boston, 2000, pp. 159–174.
- [27] S. Pastorekova, S. Parkkila, J. Pastorek, C. T. Supuran, Carbonic anhydrases: Current state of the art, therapeutic applications and future prospects, *J. Enzyme. Inhib. Med. Chem.*, **19**, 199–229 (2004).
- [28] I. Nishimori, T. Minakuchi, S. Onishi, D. Vullo, A. Cecchi, A. Scozzafava, C. T. Supuran, Carbonic anhydrase inhibitors: Cloning, characterization, and inhibition studies of the cytosolic isozyme III with sulphonamides, *Bioorg. Med. Chem.*, **15**, 7229–7236 (2007).
- [29] I. Nishimori, D. Vullo, A. Innocenti, A. Scozzafava, A. Mastrolorenzo, C. T. Supuran, Carbonic anhydrase in-

- hibitors. The mitochondrial isozyme VB as a new target for sulfonamide and sulfamate inhibitors, *J. Med. Chem.*, **48**, 7860–7866 (2005).
- [30] D. Vullo, J. Voipio, A. Innocenti, C. Rivera, H. Ranki, A. Scozzafava, K. Kaila, C. T. Supuran, Carbonic anhydrase inhibitors. Inhibition of the human cytosolic isozyme VII with aromatic and heterocyclic sulphonamides, *Bioorg. Med. Chem. Lett.*, **15**, 971–976 (2005).
- [31] C. T. Supuran, A. Scozzafava, J. Conway, *Carbonic Anhydrase: Its Inhibitors and Activators*, CRC Press, Boca Raton, 2004, pp. 25–43.
- [32] D. Vullo, M. Franchi, E. Gallori, J. Pastorek, A. Scozzafava, S. Pastorekova, C. T. Supuran, Carbonic anhydrase inhibitors: Inhibition of the tumor-associated isozyme IX with aromatic and heterocyclic sulphonamides, *Bioorg. Med. Chem. Lett.* **13**, 1005–1009 (2003).
- [33] D. Vullo, A. Innocenti, I. Nishimori, J. Pastorek, A. Scozzafava, S. Pastoreková, C. T. Supuran, Carbonic anhydrase inhibitors. Inhibition of the transmembrane isozyme XII with sulfonamides—a new target for the design of antitumor and antiglaucoma drugs, *Bioorg. Med. Chem., Lett.* **15**, 963–969 (2005).
- [34] J. Lehtonen, B. Shen, M. Vihinen, A. Casini, A. Scozzafava, C. T. Supuran, A. K. Parkkila, J. Saarnio, A. J. Kivela, A. Waheed, W. S. Sly, S. Parkkila, Characterization of CA XIII, a novel member of the carbonic anhydrase isozyme family, *J. Biol. Chem.*, **279**, 2719–2727 (2004).
- [35] I. Nishimori, D. Vullo, A. Innocenti, A. Scozzafava, A. Mastrolorenzo, C.T. Supuran, Carbonic anhydrase inhibitors: Inhibition of the transmembrane isozyme XIV with sulphonamides, *Bioorg. Med. Chem. Lett.*, **15**, 3828–3833 (2005).
- [36] H. Demirhan, M. Arslan, M. Zengin, M. Kucukislamoğlu, A Comparative Study in Oxidative Free Radical Reactions between 9-Benzylidene-9H-fluorene Derivatives and β -Dicarbonyl Compounds in the Presence of $Mn(OAc)_3$ and CAN, *Lett. In Org. Chem.*, **8**, 488–494 (2011).
- [37] R. Annunziata, V. Molteni, L. Raimondi, Synthesis and structural assignment of 2,4'-disubstituted benzylidene-fluorenes and 4'-substituted benzylidene-1-azafluorenes, *Magn. Reson. Chem.*, **36**, 520–528 (1998).
- [38] O. Arslan, B. Nalbantoglu, N. Demir, H. Ozdemir, O. I. Kufrevioglu, A new method for the purification of carbonic anhydrase isozymes by affinity chromatography, *Turk. J. Med. Sci.*, **26**, 163–166 (1996).
- [39] T. H. Maren, A simplified micromethod for the determination of carbonic anhydrase and its inhibitors, *J. Pharm. Exp. Ther.*, **130**, 2629–2634 (1960).
- [40] F. Pacchiano, M. Aggarwal, B. S. Avvaru, A. H. Robbins, A. Scozzafava, R. McKenna, C. T. Supuran, Selective hydrophobic pocket binding observed within the carbonic anhydrase II active site accommodate different 4-substituted-ureido-benzenesulfonamides and correlate to inhibitor potency, *Chem. Commun.*, **46**, 8371–8373 (2010).
- [41] K. Erol, N. Gencer, M. Arslan, O. Arslan, Purification, characterization, and investigation of in vitro inhibition by metals of paraoxonase from different sheep breeds, *Artif. Cells Nanomed. Biotechnol.*, **41**, 125–130 (2013).
- [42] N. Gencer, A. Ergun, D. Demir, In vitro effects of some anabolic compounds on erythrocyte carbonic anhydrase I and II, *J. Enzyme Inhib. Med. Chem.*, **27**, 208–210 (2012).
- [43] N. Berber, M. Arslan, E. Yavuz, C. Bilen, N. Gencer, Synthesis and Evaluation of New Phthalazine Urea and Thiourea Derivatives as Carbonic Anhydrase Inhibitors, *J. Chem.*, **2013**, 1–8 (2013).
- [44] D. Demir, N. Gencer, A. Er, Purification and characterization of prophenoloxidase from *Galleria mellonella* L. *Artif. Cells Nanomed. Biotechnol.*, **40**, 391–395 (2012).
- [45] B. Gokce, N. Gencer, O. Arslan, S. A. Turkoglu, M. Alper, F. Kockar, Evaluation of in vitro effects of some analgesic drugs on erythrocyte and recombinant carbonic anhydrase I and II, *J. Enzyme Inhib. Med. Chem.*, **7**, 37–42 (2012).

