Coumarin or benzoaxazinone bearing benzimidazolium and bis(benzimidazolium) salts; involvement in transfer hydrogenation of acetophenone derivatives and hCA inhibition

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Abstract: Four new salts of benzimidazolium and bis(benzimidazolium) which include coumarin or benzoaxazinone moieties were synthesized and the structures of the newly synthesized compounds were elucidated on the basis of spectral analyses such as ¹H-NMR, ¹³C-NMR, HSQC, IR, LC-MS and elemental analysis. Benzimidazolium salts were used intensively as N-heterocyclic carbene (NHC) precursors in the various catalytic reactions such as transfer hydrogenation (TH), C-H bond activation, Heck, Suzuki reaction etc. With the prospect of potential NHC precursor properties of the synthesized compounds, they were employed in the (TH) reaction of p-substituted acetophenones (acetophenone, p-methyl acetophenone, p-chloro acetophenone) and good yields were observed. Coumarin compounds are known as inhibitor of carbonic anhydrase and inhibition effects of the synthesized compounds on human carbonic anhydrases (hCA) were investigated as in vitro. The in vitro results demonstrated that all compounds inhibited hCA I and hCA II activity. The inhibitory effects of the synthesized compounds on the hydratase and esterase activities of these isoenzymes (esterase activity is for hCA I) were studied in vitro. In relation to these activities, the inhibition equilibrium constants (Ki) were determined. The results showed that coumarin based benzimidazolium and bisbenzimidazolium salts are more active than benzoaxazinone derivatives.

Keywords: Benzimidazolium; Benzoaxazinone; Coumarin; Carbonic Anhydrase; Transfer Hydrogenation (TH).

Introduction

Benzimidazole is a bicyclic and heterocyclic compound which consists of the fusion of benzene and imidazole rings. One of the most well known benzimidazoles in nature is N-ribosyl dimethylbenzimidazole which serves as an axial ligand for cobalt in water soluble vitamin B12¹. Because of their synthetic utility, benzimidazolide derivatives have been investigated as bioactive compounds. A number of benzimidazole derivatives were reported as, for example antihypertensive², anti-inflammatory³, antimicrobial⁴, antiviral⁵, antioxidant⁶, antitumor⁷, lipid modulator⁸ and anticoagulant agents⁹.

In recent years, in addition to their biological properties, benzimidazole derivatives were also used as a carbon scaffold for the preparation of N-heterocyclic carbene (NHC) complexes. A number of NHCs with different metal atoms were synthesized by the deprotonation of 1,3-dialkylbenzimidazolium salts¹⁰⁻¹². Moreover, quite effective catalytic systems were employed in various useful catalytic reactions using 1,3-dialkylbenzimidazolium salts and some metal salts in situ¹³⁻¹⁵. The hydrogenation of aldehydes or ketones to alcohols is an important reaction in organic synthesis. The TH reaction refers to the addition of a hydrogen molecule to another molecule from a source, such as alcohol, other than molecular hydrogen. When the TH is compared with conventional hydrogenation which uses molecular hydrogen, the experimental procedure of the TH reaction is safer and simpler than conventional hydrogenation¹⁶⁻¹⁸.

Coumarins belong to a class of compounds known as benzopyrones, some heterocyclic and bicyclic compounds which consist of the fusion of pyron and benzene rings. Some coumarin derivatives were used as anticoagulant and additives in food and cosmetics¹⁹,²⁰. In addition various biological and pharmacological properties of
coumarin derivatives were reported in the literature and most recently they were reported as inhibitors of the metalloenzyme carbonic anhydrase (CA).

As an heterocyclic compound, benzoazoxinone contains 1,4-oxazinone ring fused to benzene which has been used extensively for building bioactive compounds. The anticancer, antiulcer, antihypertensive, anti-inflammatory and other biological activities of 1,4-oxazinones and benzoxazines have been reported in the literature. In addition to these reports, the anticonvulsant activities of some benzoazinone derivatives were reported and it is known that some anticonvulsant agents inhibit human CA isoenzymes effectively.

The metalloenzyme CA catalyzes a simple but critically important physiological reaction: the involvement of the CA enzyme family, which catalyzes the physiological hydration of CO$_2$ to yield bicarbonate and a proton, in many physiological/pathological processes open up widespread opportunities for the development of diverse specific inhibitors for clinical applications.

The active site of most CAs contains a zinc(II) ion (Zn$^{2+}$), which is essential for catalysis. The CA reaction involved in many physiological and pathological processes, including the respiration and transport of CO$_2$ and bicarbonate between metabolizing tissues and the lungs, pH and CO$_2$ homeostasis; electrolyte secretion in various tissues and organs, biosynthetic reactions such as gluconeogenesis, lipogenesis and ureagenesis, bone resorption, calcification and tumorigenicity. Many of the CA isoenzymes involved in these processes are important therapeutic targets with the potential to be inhibited to treat a range of disorders including edema, glaucoma, obesity, cancer, epilepsy and osteoporosis. Given the physiological importance of the CA, the metabolic impact of chemicals for crop production should receive greater study.

The present study was carried out in order to synthesize, characterize and evaluate the catalytic and biological activities of benzimidazolium and bis(benzimidazolium) chlorides which contain coumarin or benzoazoxinone moieties. Herein, our main aim is to synthesize more effective ligands (LHX) for Ru(II) metal in the TH reaction by combining coumarin and benzoazoxinone with benzimidazolium and bisbenzimidazolium compounds. With the prospect of potential carbene properties of these functionalized salts they were employed as ligands in the TH reaction of acetophenone derivatives. The inhibitory effects of the newly synthesized compounds on the activities of purified human erythrocyte CA I and CA II isoenzymes were also investigated.

### Results and Discussion

#### Preparation and characterization of benzimidazolium and (bis)benzimidazolium salts

As mentioned in the introduction, we focused on the synthesis of the functionalized benzimidazolium and bis(benzimidazolium) salts. For this purpose, we used biologically active coumarin and benzoazoxinone groups. Firstly, we synthesized 4-chloromethyl-6,8-dimethyl-2H-chromen-2-one (1) using the procedure described by Frasin and other authors. Benzimidazolium salts (1a, 2a) were synthesized by the direct quaternization of N-alkylbenzimidazole in DMF. Bis(benzimidazolium) salts were synthesized by the reaction of 1,4-di(1H-benzo[d]imidazol-1-yl)butane with 1 or 6-(chloroacetyl)-2H-1,4-benzoxazine-3(4H)-one 2 in DMF (1/2 molar ratio, respectively). The reaction conditions for the synthesis of the four compounds were given in Scheme 1. All of the four salts are air and moisture stable in the solid state as well as in solution and they were isolated in good yields and fully characterized by $^1$H-NMR, $^{13}$C-NMR, IR, LC-MS spectroscopy and elemental analyses. We could not achieve a single crystal for any of the four compounds despite all efforts. Therefore, for further characterization of the bis(benzimidazolium) salts (1b, 2b), they were evaluated by the HSQC NMR technique. HSQC spectra were evaluated on 600 MHz NMR spectrometer to avoid problems may arise due to resolution and the spectrum of 1b is given in Figure 1. The $^{13}$C-NMR chemical shifts were consistent with the proposed structure, the imino carbon (NCHN) appeared as a typical singlet in the $^1$H-decoupled mode at 142.7, 144.1 and 144.0 ppm, respectively for benzimidazolium and bis(benzimidazolium) chlorides 1a, 2a, 1b and 2b. These values are in good agreement with the previously reported results. The $^1$H-NMR data show that the acidic -NCH$_2$- protons of the benzimidazolium salts (1a, 2a, 9.28 and 8.99, respectively) are less downfield shielded than the bis(benzimidazolium) salts (1b, 2b, 10.43 and 10.11, respectively) which indicates a considerable difference in the acidity of these protons in agreement with the literature. In the structures of the coumarin and benzoazoxinone bearing salts 1b and 2b, olefinic protons are important for characterization and their signals were observed were ranged from 5.91 to 5.99 ppm in $^1$H-NMR, olefinic carbons were observed in the scale of 112.2 and 112.7 ppm in $^{13}$C-NMR as a singlet for 1a and 1b, respectively. For benzoazoxinone bearing salts 2a and 2b, free -N-H protons were located in the range of 11.09 and 11.17 ppm, respectively. IR spectra and elemental analysis also matched the structures of compounds 1a-2b.
Scheme 1. Synthesis of benzimidazolium and bis(benzimidazolium) salts 1a,b and 2a,b

Figure 1. HSQC spectrum of compound 1b
**Catalytic Hydrogen Transfer Reaction**

Catalytic studies with 1a-2b (LHX) were performed for the TH of acetophenone to give 1-phenylethanol in the presence of base with 2-propanol as a hydrogen source (Scheme 2). The reaction conditions for this important process are economic, relatively mild and environmentally friendly. The volatile acetone product can also be easily removed to shift an unfavorable equilibrium.

The acetophenone was kept as a test substrate and was allowed to react in 2-propanol with catalytic quantities of Ru/LHX in the presence of different bases like KOH, NaOH, Et₃N (as organic base) and KO'Bu. It has been observed that NaOH and KOH have good conversion when compared to Et₃N and KO'Bu in TH reactions. According to the high rate of conversion, the bases are classified: KOH > NaOH > KO'Bu > Et₃N. As in previous studies, the best results were obtained with KOH (Table 1).

Thus, the catalytic experiments were carried out using 10 mmol of acetophenone derivatives, 0.01 mmol of [(p-cymene)RuCl₂], 0.02 mmol of LHX, 5 mmol of KOH, and 10 mL of 2-propanol, with a catalyst-base-substrate ratio of 0.02: 1: 10.

![Scheme 2. Hydrogen transfer of some p-substitute acetophenone derivatives by [(p-cymene)RuCl₂]₂/LHX catalyst system.](image)

**Table 1.** Catalytic activity for transfer hydrogenation of acetophenone catalyzed by Ru(II)/LHX catalyst system with different bases.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Ru(II)/LHX</th>
<th>Base</th>
<th>Yield (%)</th>
<th>TOF (h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(1a)</td>
<td>KOH</td>
<td>17.2, 22.0, 32.1, 48.4, 63.8</td>
<td>516</td>
</tr>
<tr>
<td>2</td>
<td>(2a)</td>
<td>KOH</td>
<td>15.0, 18.4, 28.1, 45.7, 61.2</td>
<td>450</td>
</tr>
<tr>
<td>3</td>
<td>(1b)</td>
<td>KOH</td>
<td>15.1, 20.3, 30.2, 49.0, 64.9</td>
<td>453</td>
</tr>
<tr>
<td>4</td>
<td>(2b)</td>
<td>KOH</td>
<td>19.2, 25.0, 34.5, 49.7, 69.1</td>
<td>576</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>KOH</td>
<td>14.0, 17.8, 27.3, 45.0, 59.4</td>
<td>420</td>
</tr>
<tr>
<td>6</td>
<td>Only KOH</td>
<td></td>
<td>9, 14</td>
<td>78</td>
</tr>
<tr>
<td>7</td>
<td>(1a)</td>
<td>NaOH</td>
<td>7.5, 12.4, 20.6, 26.3, 30.2</td>
<td>225</td>
</tr>
<tr>
<td>8</td>
<td>(2a)</td>
<td>NaOH</td>
<td>8.2, 13.6, 22.5, 27.9, 32.5</td>
<td>246</td>
</tr>
<tr>
<td>9</td>
<td>(1b)</td>
<td>NaOH</td>
<td>8.9, 15.1, 22.8, 28.2, 33.1</td>
<td>267</td>
</tr>
<tr>
<td>10</td>
<td>(2b)</td>
<td>NaOH</td>
<td>10.1, 17.6, 25.6, 28.4, 35.5</td>
<td>303</td>
</tr>
<tr>
<td>11</td>
<td>(1a)</td>
<td>Et₃N</td>
<td>&lt;5</td>
<td>n.c.</td>
</tr>
<tr>
<td>12</td>
<td>(2a)</td>
<td>Et₃N</td>
<td>&lt;5</td>
<td>n.c.</td>
</tr>
<tr>
<td>13</td>
<td>(1b)</td>
<td>Et₃N</td>
<td>&lt;5</td>
<td>n.c.</td>
</tr>
<tr>
<td>14</td>
<td>(2b)</td>
<td>Et₃N</td>
<td>&lt;5</td>
<td>n.c.</td>
</tr>
<tr>
<td>15</td>
<td>(1a)</td>
<td>KO'Bu</td>
<td>5.2, 9.3, 13.5, 19.1, 22.4</td>
<td>156</td>
</tr>
<tr>
<td>16</td>
<td>(2a)</td>
<td>KO'Bu</td>
<td>5.1, 10.0, 15.1, 21.0, 25.3</td>
<td>153</td>
</tr>
<tr>
<td>17</td>
<td>(1b)</td>
<td>KO'Bu</td>
<td>5.0, 9.7, 14.5, 20.2, 24.7</td>
<td>150</td>
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<td>18</td>
<td>(2b)</td>
<td>KO'Bu</td>
<td>5.2, 10.9, 18.1, 23.2, 27.1</td>
<td>156</td>
</tr>
<tr>
<td>19</td>
<td>(2b)</td>
<td>KOH</td>
<td>31</td>
<td>155</td>
</tr>
<tr>
<td>20</td>
<td>(2b)</td>
<td>KOH</td>
<td>17</td>
<td>85</td>
</tr>
<tr>
<td>21</td>
<td>(2b)</td>
<td>Absence of base</td>
<td>&lt;5</td>
<td>n.c.</td>
</tr>
</tbody>
</table>

**Reaction conditions:** 10.0 mmol acetophenone, 5 mmol KOH, 0.02 mmol Ru(II)/LHX, 2-propanol (6 mL); Temperature 80 °C. S/C (500/1).

1. GC yields, yields are based on phenylethanol,
2. TOF = moles of product / (moles of the catalyst)x(hour),
3. n.c.: not calculated.
4. a 10 min., d 30 min., d 60 min., e 120 min., f 180 min.

Under these conditions p-chloroacetophenone reacts very well and in good yields with 2-propanol (Figure 2). The presence of electron withdrawing substituent (Cl) on acetophenone has a significant effect on the reduction of p-substitute acetophenone derivatives to their corresponding alcohols. Among the tested ligands, 2b is highly efficient in the transfer hydrogenation of ketones to secondary alcohols.
All the experiments were carried out in an air atmosphere. This indicates that air is not involved in the TH process and the [(p-cymene)RuCl₂]₂/LHX catalyst systems are air-stable.

![Graph](image)

**Figure 2.** Catalytic activity for the transfer hydrogenation of acetophenone ♦ for (1a) appear as blue, ■ for (2a) appear as red, ▲ for (1b) appear as green, x for (2b) appear as purple.
(a) In the presence of 2-propanol at 80 °C with [(p-cymene)RuCl₂]₂/LHX; methylacetophenone/Ru/KOH, 10:0.02:1
(b) In the presence of 2-propanol at 80 °C with [(p-cymene)RuCl₂]₂/LHX; 4-chloroacetophenone/Ru/KOH, 10:0.02:1

**CA Inhibition**

The recently reported class of effective CAIs, coumarins and thiocoumarins, have an inhibition mechanism not dependent on Zn(II), and bind (in hydrolyzed form) to the same active site region as the activators, occluding the entrance to the active site while the other three CAI groups bind to the Zinc (II) ion or Zinc coordinated water molecule/hyroxide ion. Crystallographic studies showed that the natural coumarin compound, namely 6-(1S-hydroxy-3-methylbutyl)-7-methoxy-2H-chromen-2-one, was hydrolyzed within the CA active site with the formation of 2-hydroxycinnamic acids, which have significant CA inhibitory properties and bind in a completely unprecedented manner to the enzyme, not interacting with the Zn(II) ion.

To evaluate the CA inhibitory activity, all of the compounds were subjected to CA inhibition assay with CO₂ as substrate. The results demonstrated that all compounds inhibited hCA I and II enzyme activity. The inhibition values of 1-2b against CAs are summarized in Table 2. We determined IC₅₀ values ranging between 5.55-19.98 µM for hCA I and 6.06-25.23 µM for hCA II and Ki values for hCA I esterase activity were ranged between 55.23-252.21 µM. Esterase activity experiments showed that inhibition type of these compounds is non-competitive. Among them, 1a and 1b was found to be the most active. From this study, some fundings emerged and the quaternization of coumarin and benzoxazinone compounds with benzimidazole or...
bis(benzimidazole) increased the inhibitory activity. When the activities of the synthesized compounds were compared, the coumarin bearing salts were found to be more active than the benzoxazinone bearing salts, especially for hCA II (1b > 1a > 2a > 2b).

Most recently Korkmaz and co-workers reported the synthesis of thiourea containing benzimidazole moieties and they tested three derivatives of these compounds as inhibitors of hCA I and hCA II\(^9\). Their K\(_i\) values were ranged between 46.1-73.6 \(\mu M\) for hCA I. In this study, compounds 1a,1b and 2a showed close inhibitory activity when compared benzimidazole-thioure hybrid compounds.

**Table 2.** The IC\(_{50}\) and Ki values of compounds (hCA, human carbonic anhydrase)

<table>
<thead>
<tr>
<th>Compound</th>
<th>hCA I Hydratase activity IC(_{50}) ((\mu M))</th>
<th>hCA II Hydratase activity IC(_{50}) ((\mu M))</th>
<th>hCA I Esterase activity Ki ((\mu M))</th>
<th>Inhibition type</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10.04</td>
<td>14.24</td>
<td>252.21</td>
<td>Non-competitive</td>
</tr>
<tr>
<td>2</td>
<td>19.98</td>
<td>25.23</td>
<td>167.82</td>
<td>Non-competitive</td>
</tr>
<tr>
<td>1a</td>
<td>6.14</td>
<td>6.66</td>
<td>55.23</td>
<td>Non-competitive</td>
</tr>
<tr>
<td>1b</td>
<td>5.55</td>
<td>6.06</td>
<td>61.33</td>
<td>Non-competitive</td>
</tr>
<tr>
<td>2a</td>
<td>6.59</td>
<td>21.07</td>
<td>87.41</td>
<td>Non-competitive</td>
</tr>
<tr>
<td>2b</td>
<td>11.20</td>
<td>23.04</td>
<td>171.71</td>
<td>Non-competitive</td>
</tr>
</tbody>
</table>

**Conclusion**

In conclusion, we have reported the synthesis and characterization of novel functionalized benzimidazolium and bis(benzimidazolium) salts. These compounds are hybrid substrates which include benzimidazolium as a NHC precursor and coumarin as a CA inhibitor. It is known that a mixture of azolium salts and \([p(\text{cymene})\text{RuCl}_2]\); has been used as a precatalyst for the TH reaction. One of the main aims was to investigate the effects of coumarin and benzoxazinone groups as ligands in the TH reaction. Thus, the catalytic activities of the synthesized salts were investigated in the TH reaction and good yields were obtained. With the prospect of potential CA inhibitory properties of the synthesized compounds, they were investigated as CA inhibitors and the results showed that all of the synthesized compounds inhibited hCA I and hCA II enzyme activity. The contribution of benzimidazolium salts to CA inhibitory activity was investigated. Besides coumarin bearing salts (1a,b), benzoxazinone bearing salts (2a,b) inhibited hCA I and hCA II effectively. Our results suggest that these newly synthesized compounds are likely to be adopted as candidates to treat glaucoma and epilepsy.

**Experimental Section**

For the preparation of benzimidazolium and bisbenzimidazolium salts, all reactions were carried out in standard Schlenk type flasks. Chemicals and solvents were purchased from Sigma Aldrich, Merck. 6-(chloroacetyl)-2H-1,4-benzoxazine-3(4H)-one was supplied commercially, controlled by \(^1\)H-NMR and used without further purification. Melting points were determined by Electrothermal-9200 melting point apparatus. The FT-IR spectra were recorded on an ATR unit in the range of 400-4000 cm\(^{-1}\) with a Perkin Elmer Spectrum 100 Spectrophotometer. The \(^1\)H-NMR and \(^13\)C-NMR spectra were recorded using a Bruker FT spectrometer operating at 300.13 MHz (\(^1\)H), 75.47 MHz (\(^13\)C) and HSCQ spectra were recorded using Bruker 600 Avance 3 HD spectrometer operating at 600.134 MHz (\(^1\)H) and 150.918 MHz (\(^13\)C). Chemical shifts are given in ppm relative to TMS. Elemental analyses were performed by a LECO CHNS-932 elemental analyzer at IBTAM (Inonu University Scientific and Technological Research Center). LC-MS spectra were performed on an Agilent 1100 LC/MSD SL mass spectrometer equipped with an electrospray ion source.

**Synthesis and characterization of compounds**

**Synthesis of 1-(2,3,4,5,6-pentamethylbenzyl)benzimidazol and 1,4-di(1H-benzo[d]imidazol-1-yl)butane.** 1-(2,3,4,5,6-pentamethylbenzyl)benzimidazol and 1,4-di(1H-benzo[d]imidazol-1-yl)butane were synthesized by the procedure described in the literature\(^50\).

**Synthesis of benzimidazolium salts (1a and 2a)** (General Method): 4-chloromethyl-6,8-dimethyl-2H-chromen-2-one (compound 1) was synthesized by the procedure described by Frasinsky (m.p.:146 ºC ref. no 48 143-145 ºC). 1-(2,3,4,5,6-pentamethylbenzyl)benzimidazol (1.4 g, 5 mmol) was dissolved in 5 mL DMF. Five millimoles of 4-chloromethyl-6,8-dimethyl-2H-chromen-2-one (1.12 g) (1) or 6-chloroacetyl-2H-1,4-benzoxazine-3(4H)-one (1.13 g) (2) was added to this solution and the resulting mixture was heated for 24 hours at 80 ºC. Later, the mixture was cooled to ambient temperature. Twenty milliliters of diethyl ether was added and the precipitates were collected by filtration. The crude product was washed with hexane (2x10 mL) and diethyl ether(10 mL), then dried under reduced pressure.
1-(2,3,4,5,6-pentamethylbenzyl)-3-((6,8-dimethyl-2H-chromen-2-one-4-yl)methyl)benzimidazolium chloride (1a). Yield 2.3 g, 92%, white solid, mp 257 °C. IR spectrum, ν, cm⁻¹: 1731(CO); 1631(C=C); 1590(C=C). ¹H-NMR (300 MHz, DMSO-d₆), δ, ppm: 9.28(s, 1H, H); 8.34-7.74(m, 6H, H₆); 6.10(s, 2H, H₃); 5.91(s, 1H, H); 5.78(s, 2H, H₁); 2.38(s, 3H, CH₃); 2.36(s, 3H, HCH₂); 2.26(s, 3H, HCH₂); 2.24(s, 6H, HCH₂); 2.23(s, 6H, HCH₂). ¹³C-NMR (75 MHz, DMSO-d₆), δ, ppm: 159.9(C₈); 149.8; 149.7; 142.7(C₂); 136.8; 135.1; 134.4; 133.8; 133.5; 132.3; 132.2; 127.8; 127.4; 126.1; 125.9; 122.4; 116.8; 114.7; 114.4; 112.2(C₄); 47.3(C₁); 47.1(C₁). Found, %: C 74.1; H 6.53; N 5.66. C₃₉H₃₅ClO₅N₂. Calculated, %: C 74.31; H 6.64; N 5.59.

1-(2,3,4,5,6-pentamethylbenzyl)-3-(6-acetyl-2H-1,4-benzoxazine-3(4H)-one)benzimidazolium chloride (2a). Yield 2.06 g, 86%, white solid, mp 210 °C. IR spectrum, ν, cm⁻¹: 3426(NH); 1692(CO); 1598(C=C). ¹H-NMR (300 MHz, DMSO-d₆), δ, ppm: 11.10(s, 1H, H); 8.99(s, 1H, H); 8.31-7.15(m, 7H, H₆); 6.21(s, 2H, H₃); 5.81(s, 2H, H); 4.74(s, 2H, H₁); 2.25(s, 3H, HCH₂); 2.22(s, 12H, HCH₂). ¹³C-NMR (75 MHz, DMSO-d₆), δ, ppm: 190.1(C₁); 164.4(C₄); 148.6; 142.7(C₃); 136.9; 134.3; 133.5; 132.8; 131.5; 128.5; 128.0; 127.5; 127.1; 126.0; 125.1; 116.8; 115.7; 114.4; 67.2(C₁). Found, %: C 69.33; H 6.19; N 8.51. C₃₉H₃₃ClO₅N₂. Calculated, %: C 69.11; H 6.00; N 8.34.

Synthesis of bis(benimidazolium) salts (1b and 2b) (General Method). 1,4-di(1H-benzo[d]imidazol-1-yl)butane (0.87 g, 3mmol) was dissolved in 5 mL DMF. Six millimoles of 4-chloromethyl-6,8-dimethyl-2H-chromen-2-one (1.34 g) (1) or 6-chloroacetyl-2H-1,4-benzoxazine-3(4H)-one (1.36 g) (2) was added to this solution and the resulting mixture was heated for 48 hours at 90 °C. After this period, the mixture was cooled to ambient temperature. Twenty milliliters of diethyl ether was added and the precipitates were collected by filtration. The crude product was washed with hexane (2x10 mL) and acetone (10 mL) then dried under reduced pressure.

1,4-Bis-((6,8-dimethyl-2H-chromen-2-one-4-yl)methyl)benzimidazolium-3(4H)-yl)butane dichloride (1b). Yield 1.4 g, 64%, white solid, mp 222 °C. IR spectrum, ν, cm⁻¹: 1717(CO); 1589(C=C). ¹H-NMR (600 MHz, DMSO-d₆), δ, ppm: 10.43 (s, 1H, H); 8.26-7.45(m, 6H, H₆); 6.28(s, 2H, H₁); 5.97(s, 1H, H); 4.70(s, 2H, H); 2.51(s, 3H, HCH₂); 2.41(s, 3H, HCH₂); 2.15(s, 2H, H). ¹³C-NMR (150 MHz, DMSO-d₆), δ, ppm: 159.9(C₁); 149.9; 149.4; 144.1(C₄); 135.2; 133.8; 131.9; 131.7; 127.5; 127.3; 125.8; 122.5; 116.8; 114.6; 114.2; 112.7(C₄); 47.0(C₄); 46.9(C₄); 25.7(C₁). Found, %: C 68.23; H 5.53; N 7.46. C₃₀H₂₆Cl₂O₄N₄. Calculated, %: C 68.57; H 5.48; N 7.62.

1,4-Bis-((6-acetyl-2H-1,4-benzoxazine-3(4H)-one)benzimidazolium-3-yl)butane dichloride (2b). Yield 1.53 g, 69%, mp 270 °C. IR spectrum, ν, cm⁻¹: 3376(NH); 1674(CO); 1601(C=N). ¹H-NMR (600 MHz, DMSO-d₆), δ, ppm: 11.15(s, 1H, H); 10.11(s, 1H, H); 8.20-7.18(m, 7H, H₆); 6.43(s, 2H, H); 4.75(s, 4H, H and H); 2.08(s, 2H, H). ¹³C-NMR (150 MHz, DMSO-d₆), δ, ppm: 190.0(C₁); 164.4(C₄); 148.7; 144.0(C₃); 132.5; 131.1; 128.5; 128.1; 127.3; 127.1; 125.3; 116.8; 115.9; 114.5; 114.3; 67.3(C₄); 53.3(C₃); 46.6(C₄); 25.9(C₂). Found, %: C 61.34; H 4.43; N 11.47. LC-MS (m/z):705.2 [MH⁺]. C₃₉H₃₃ClO₄N₉. Calculated, %: C 61.54; H 4.62; N 11.33.

General procedure for transfer hydrogenation reaction (General Method). In a typical experiment, 0.01 mmol (6.1 mg) of [p-cymene]RuCl₃ with 0.02 mmol of benzimidazolium or bis(benimidazolium) chlorides, 10 mmol of p-substituted acetophenone derivatives and 5 mmol (280 mg) of KOH were heated at 80 °C for a period of 180 min in 2-propanol (6 mL) as a hydrogen source. After the desired reaction time, the mixture was diluted with diethyl ether (2 mL) and filtered from a mini-column. The purity of the compounds was checked by GC. The yields obtained were related to the residual unreacted acetophenone. The reactions were conducted at a (S/C/base) molar ratio of 10: 0.02: 1.

Carboxic anhydrase inhibition

Preparation of hemolysate and purification from blood red cells

Blood samples (25 mL) were taken from healthy volunteers. They were anticoagulated with acid-citrate-dextrose, centrifuged at 2000 g for 20 min at 4 °C and the supernatant was removed. The packed erythrocytes were washed three times with 0.9 % NaCl and then hemolyzed in cold water. The ghosts and any intact cells were removed by centrifugation at 2000 g for 25 min at 4 °C, and the pH of the hemolysate was adjusted to pH 8.5 with solid Tris-base. The 25 mL hemolysate was applied to an affinity column containing L-tyrosine-sulfonamide-Sepharose-4B51 equilibrated with 25mM Tris-HCl / 0.1M Na₂SO₄ (pH 8.5). The affinity gel was washed with 50mL 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 hCA-II respectively. Fractions of 3 mL were collected and their absorbance measured at 280 nm.

Hydratase activity assay

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 hydration52. 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 \(t_0 - t/t_c\) where \(t_0\) and \(t_c\) are the times for pH change of the nonenzymatic and the enzymatic reactions, respectively.

**Esterase activity assay**

Carbonic anhydrase activity was assayed by following the change in absorbance at 348 nm of 4-nitrophenyl-acetate (NPA) to 4-nitrophenolate ion over a period of 3 min at 25°C using a spectrophotometer (HACH RANGE DV 6000 UV-VIS) according to the method described in the literature. The enzymatic reaction, in a total volume of 3.0 mL, contained 1.4 mL of 0.05 M Tris-SO₄ buffer (pH 7.4), 1 mL of 3 mM 4-nitrophenylacetate, 0.5 mL H₂O and 0.1 mL enzyme solution. A reference measurement was obtained by preparing the same cuvette without enzyme solution. The inhibitory effects of the synthesized compounds were examined. All compounds were tested in triplicate at each concentration used. Different concentrations of the compounds were used.

**In vitro inhibition studies**

For the inhibition studies of the synthesized compounds, different concentrations of compounds were added to the enzyme. The activity percentage values of CA for different concentrations of each coumarin and benzoxazinone derivative were determined by regression analysis using Microsoft Office 2000 Excel. CA enzyme activity without compounds 1-2b solution was accepted as 100 % activity.

**References**