Carbonic Anhydrase Activators. VII.[1] Isozyme II Activation by Bisazolyl-methanes, -ethanes and Related Azoles

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A correlation was found between the carbonic anhydrase II activating power and the pK_a values for a series of azoles, bisazolylmethanes and bisazolylethanes. Strong activations were found for compounds with pK_a's in the interval 6.5—8.0. The mechanism of action for such activators is discussed.

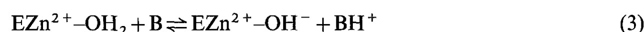
Keywords carbonic anhydrase; isozyme II; azole; bisazolyl-methane/ethane; pK_a

Carbonic anhydrase (CA, EC 4.2.1.1) catalyzes a very simple physiological reaction, the interconversion between carbon dioxide and hydrogen carbonate anion.[2]

$$CO_2+2H_2O \rightleftharpoons H_3O^+ + HCO_3^- \quad (1)$$

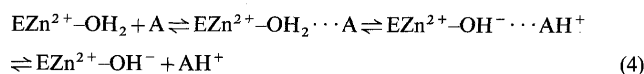
CA inhibitors of the sulfonamide type[3] are widely used pharmaceuticals in the treatment of a variety of disorders such as glaucoma,[4] epilepsy,[5] gastro-duodenal ulcers,[6] *etc.* Activators of this enzyme were only recently reported[7,8] and their mechanism of action, mainly for isozymes CA I and CA II, was investigated.[9] These activators act in the catalytic cycle by forming a complex with the enzyme and facilitating an 'intermolecular' proton transfer step (which is the rate-determining process in the catalytic turnover)[10]:

$$EZn^{2+}\text{–}OH^- + CO_2 \rightleftharpoons EZn^{2+}\text{–}HCO_3^- \overset{H_2O}{\rightleftharpoons} EZn^{2+}\text{–}OH_2 + HCO_3^- \quad (2)$$



$$EZn^{2+}\text{–}OH_2 + B \rightleftharpoons EZn^{2+}\text{–}OH^- + BH^+ \quad (3)$$

In Eq. 3, B represents a buffer which can be either an amino acid residue within the enzyme active site (such as His 64)[10] or an external buffer molecule present in the environment. In the presence of activators (A), Eq. 3 becomes[9]:



$$EZn^{2+}\text{–}OH_2 + A \rightleftharpoons EZn^{2+}\text{–}OH_2 \cdots A \rightleftharpoons EZn^{2+}\text{–}OH^- \cdots AH^+ \rightleftharpoons EZn^{2+}\text{–}OH^- + AH^+ \quad (4)$$

enzyme–activator complexes

As shown in previous papers[1,7,9] activators of isozymes I and II must possess protonable groups (generally, primary, secondary or tertiary amino groups) attached to a bulky aromatic or heterocyclic moiety. Effective activation was achieved with histamine **1**,[7a,9] 2-(β-aminoethyl)-5-amino-1,3,4-thiadiazole **2**,[7a] as well as β-aminoethyl-substituted pyridinium cations **3**,[1] among others. Although a QSAR (quantitative structure activity relationships) study is available for CA II activators,[11] pK_a values were not included among the parameters used in the calculations due to the lack of such data for the investigated compounds.

In this paper we report rather efficient CA II activators, derivatives of various azoles, bisazolylmethanes and -ethanes, as well as the first correlation between the activation efficiency and the corresponding pK_a values. These experiments provide new insights regarding the CA mechanism of action/activation and could explain the physiopathological significance of CA activation.

Materials and Methods

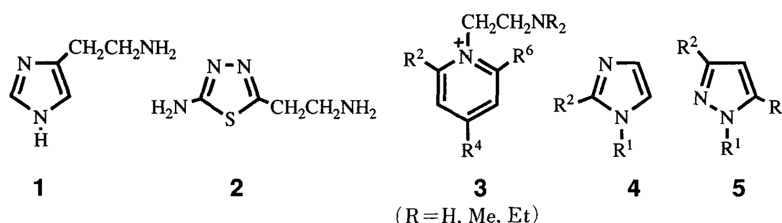
Compounds **4** and **5** were commercial (from Merck or Fluka) except for **5b** which was prepared according to literature procedures.[12] Compounds **6**—**8** were prepared as described in an earlier paper.[13] Bovine isozyme CA II was from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Assay for Activating Effects of Compounds 4—8 The activators were tested using Maren's micromethod[14] and the SEI (substrate–enzyme–inhibitor technique) at 0 °C. With this technique, the enzyme is added to the assay cell first (through which a constant flow of 140 ml/min CO_2 is bubbled), followed by the activator, and equilibrium is reached afterwards. Stock solutions of activator were prepared in distilled, deionized water (10^{-2} M), with possible addition of 10% dimethyl sulfoxide (DMSO) (for

TABLE I. CA II Activation with Compounds **4** and **5** (at 10^{-5} M)

Compd.	R^1	R^2	R^3	pK_a[a)]	% activation[b)]
4a	H	H	—	7.08[c)]	190±2.3
4b	Me	H	—	7.12[d)]	194±5.1
4c	Et	H	—	7.19[d)]	203±3.8
4d	Me	Me	—	8.00[d)]	247±7.0
5a	H	H	H	2.50[c)]	100±3.9
5b	Me	H	H	2.06[d)]	100±3.8
5c	Me	Me	Me	3.74[d)]	118±4.1

a) The pK_a values are known at ±0.05 pK_a units. *b)* Control CA activity in the absence of activator is (100±4.3)% (20 determinations), for all the other data the values correspond to 5 determinations. *c)* Ref. 16. *d)* Ref. 17.



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compounds possessing a poor water solubility). Dilution until 10^{-8} M was done from then on with distilled deionized water. All compounds were tested in the concentration range 10^{-8}—10^{-3} M.

In a special CO_2 bubbler cell, 0.2 ml of distilled water was added, followed by 0.1 ml of 0.1 M Na_2SO_4 solution (in order to maintain the ionic strength constant) and 0.4 ml of phenol red indicator solution, 0.1 ml of activator solution and 0.1 ml of CA solution (approximately, 2 enzyme units; enzyme concentration in the assay cell was near 5×10^{-9} M). The hydration reaction was initiated by addition of 0.1 ml of buffer solution (0.3 M Na_2CO_3 and 0.2 M $NaHCO_3$). The time to obtain a color change (pH 7.2) was recorded with a stopwatch. The enzyme specific activity (SA) was calculated using the formula[14]: $SA = 2000\ (T_0 - T)/T$, where T_0 is the time necessary for color change for the uncatalyzed reaction and T, the time necessary for color change in the presence of the activator. The enzyme specific activity in the absence of activators was taken as 100%. The values of Tables I and II are the mean from at least five determinations.

Results and Discussion

The first series of compounds of types **4** and **5** which were investigated are gathered in Table I. CA II activation by imidazole **4a** was reported by several groups,[9,10,15] which used different assay methods and enzymes of diverse origin (human, bovine, equine, *etc*.). On the other hand, one of us[9] showed that pyrazole **5a** is totally devoid of such property. Thus, it appeared of interest to investigate the effect on CA II of different imidazole and pyrazole derivatives possessing pK_a values in the range of 2.0—8.0 pK_a units.

Table II. CA Activation with Compounds **6**—**8** (at 10^{-5} M)

Compd.	n	R^1	R^2	R^3	pK_{a1}[a]	pK_{a2}[a]	% activation[b]
6a	1	H	—	—	3.76	5.56	140±4.5
6b	1	Me	—	—	4.97	6.64	169±4.8
6c	2	H	—	—	4.87	6.41	154±5.2
6d	2	Me	—	—	5.83	7.28	231±6.3
7	2	—	—	—	3.71	4.61	115±1.9
8a	1	H	H	H	−2.12	0.12	100±3.1
8b	2	H	H	H	−0.24	1.67	100±4.2
8c	1	Me	H	Me	0.37	2.14	100±1.7
8d	2	Me	H	Me	1.30	3.39	112±2.5
8e	2	H	Br	H	−1.90	0.00	100±2.3

a) Ref. 13. These values are known with an error of ±0.05 pK_a units. *b*) Control CA activity in the absence of activator is (100±4.3)% (20 determinations), for all the other data the values correspond to 5 determinations.

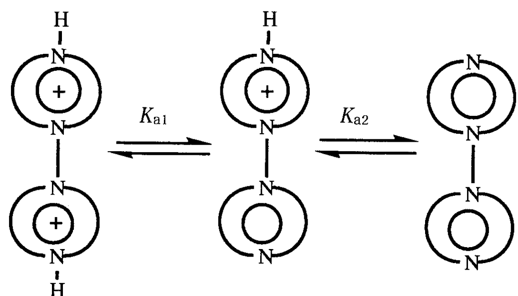


Chart 1

Data of Table I (obtained at 10^{-5} M concentrations) show that imidazoles **4** are strong activators while pyrazoles **5** are either totally devoid of activity or are weak activators and that for each group of compounds, imidazoles and pyrazoles, the activating efficiency increases with increasing pK_a. A pK_a value near 7 seems necessary for this action, an observation which may be correlated with the fact that the pK_a of water coordinated to Zn(II) within the CA active site is 7.6±0.6.[10] Unfortunately, compounds possessing pK_a's in the 4—6 range were not yet available in these series (the only derivative with a pK_a near 4, **5c**, is a weak activator).

Recently, a series of pK_a's for bisazolylmethanes and bis-azolylethanes were reported.[13] In contrast to compounds **4** and **5**, these derivatives are difunctional acids on protonation and possess a large range of acidities (values in the interval −2.1 to +7.3, as seen in Table II). The acid–base equilibria for compounds **6**, **7** and **8** are shown in Chart 1.

In Table II are reported the activation experiments (again, at 10^{-5} M concentrations) for these last compounds. Similarly to imidazoles **4**, strong activations are observed for bisimidazole derivatives which possess pK_a values in the range 6.0—7.3 (compounds **6b**—**d**). Derivative **6a** (pK_a around 5.5) has an intermediate potency between the strong activators (**6b**—**d**) and the weak activators, the bis-benzimidazole derivative **7** and bispyrazole **8d**, both having pK_a's in the range 3.4—4.6. The very weak bases, **8a**, **8b**, **8c** and **8e** are devoid of activating power.

Activation by these derivatives depends on their concentration in the assay cell (for each compound, ex-

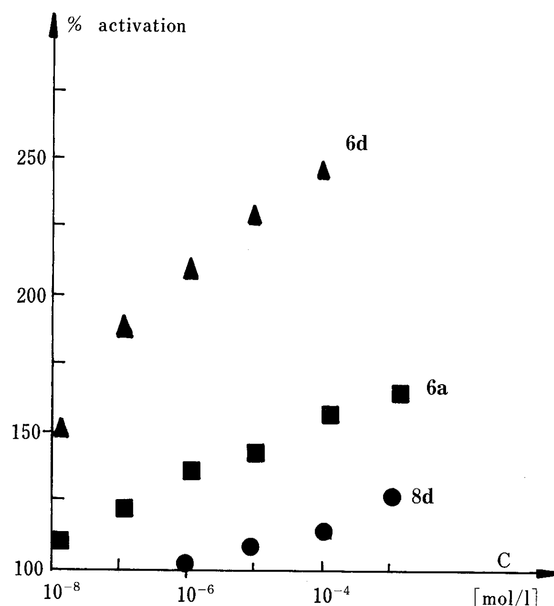
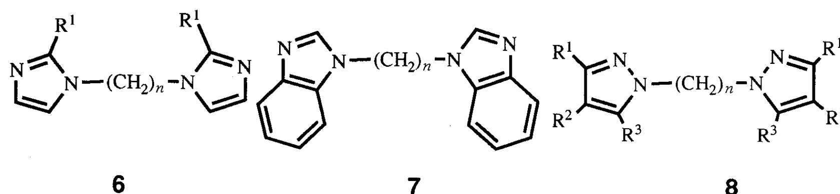


Fig. 1. Activation Plots (% Activation *vs*. Molarity of Activator) of CA II for Compounds **6a**, **6d** and **8d**



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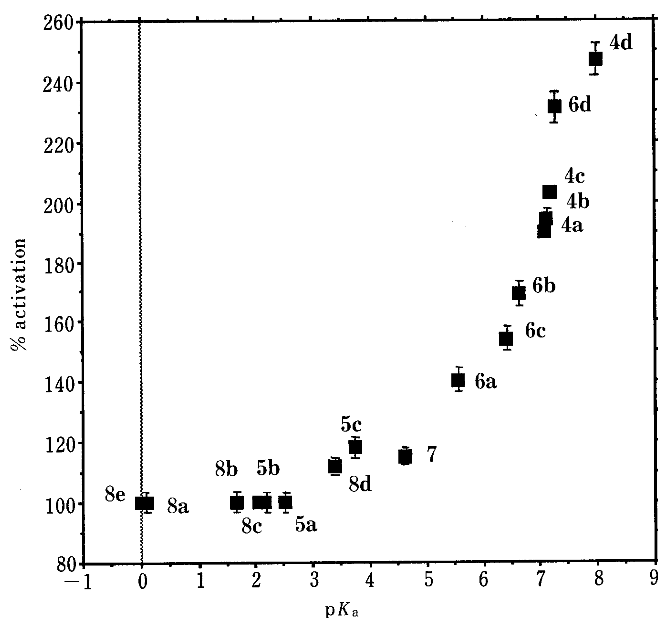


Fig. 2. Plot of CA II Activation *vs.* pK_a of Activator (at 10^{-5} M Concentrations of Activator) for All Compounds of Tables I and II

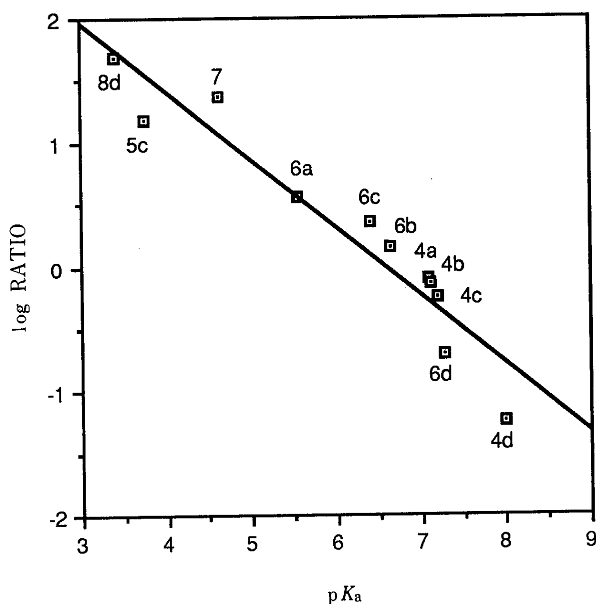
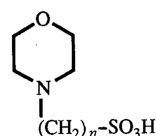


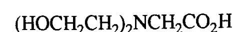
Fig. 3. Plot of log RATIO *vs.* pK_a for All the Active Compounds
log RATIO $=3.604-0.5488pK_a$. $R=0.95$.

periments were carried out in the 10^{-8}—10^{-3} mol/l concentration range, see Materials and Methods for details). In Fig. 1 are shown activation curves at different concentrations for a very strong (**6d**), a moderate (**6a**) and a weak (**8d**) activator. In all cases, increasing concentrations of activator produced an increased activation of CA.

In Fig. 2 we present a plot of activation efficiency (at 10^{-5} M concentration) *vs.* pK_a values of the activators (the highest pK_a was used for dibasic compounds **6**—**8**). If we assume that these data correspond to a sigmoid curve, then we can use the transformation RATIO $=(\%A_{max}-\%A_i)/(\%A_i-\%A_{min})$ and represent log RATIO *vs.* pK_a. After several attempts, an acceptable fit was found for $\%A_{max}=255$ and $\%A_{min}=109$. A representation of the plot, excluding the inactive compounds, **5a**, **5b**, **8a**, **8b**, **8c** and **8e**, is given in Fig. 3. If this corresponds to the experimental reality, no activators capable of more than 255% of activation will be found.



9, $n=3$, MOPS
10, $n=2$, MES



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It is interesting that compounds from different classes of heterocycles (imidazoles, benzimidazoles, pyrazoles as well as their bis-derivatives) behave similarly. Mention should be made of the fact that the pK_a is not the only factor influencing the activation power. For instance, other compounds unrelated to **4**—**8**, but with pK_a's in the 6—8 units range, such as the buffers MOPS [4-morpholinepropanesulfonic acid **9** ($pK_a=7.2$)], MES [4-morpholine-ethanesulfonic acid **10** ($pK_a=6.1$)] and bicine [*N*,*N*-bis(2-hydroxyethyl)glycine **11** ($pK_a=8.3$)] are ineffective as CA activators.[9] Another interesting observation is that bis-azolylmethanes and -ethanes behave like normal azoles; moreover, as Figs. 2 and 3 clearly show, only the pK_a corresponding to the first protonation is representative of their behavior. That could signify that only one azol-1-yl residue is bound to the active site of CA, but this is only a hypothesis.

Thus, precise electronic and steric requirements are needed for a compound to act as a CA activator. What the present study stressed is the fact that in a series in which activation action was detected, such as the azoles investigated here, efficiency and fine-tuning of the process can be controlled by designing compounds with certain pK_a values. We have found that for an azole to be a very efficient activator it has to have pK_a's between 6.5 and 8. It remains to be determined if pK_a's over 8[18] maintain or slightly increase the potency [as Fig. 3 suggests] or if too strongly basic compounds show a decrease in efficiency (bell-shaped curve).

The mechanism of action of these activators involves a facilitated proton transfer step, assisted by the heterocyclic compound. An activator possessing a protonable group with a suitable pK_a (about 7), already bound within the active site, may engage in hydrogen bonding which stabilizes the transition state. The overall effect is that the proton transfer is facilitated because two pathways are available, the normal one, involving the active site residue His 64, and another one provided by the activator bound within the active site. Thus, we conclude that both the basicity range and the structure of the activator are important requirements for a compound to have access to the CA active site and to shuttle protons during the catalytic turnover.

What is the significance of such observations? Maybe to develop agents that improve the action of CA in abnormal states. Sly *et al.*[19] reported that in people with CA II deficiency syndrome, clinical signs such as osteopetrosis, renal tubular acidosis and brain calcifications occur. In such cases, when other CA isozymes are present (which possess, however, a weaker activity as compared to the perfectly evolved protein which is CA II),[20] compounds able to activate these isozymes might be beneficial for healing

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these metabolic disorders of bone, brain and kidney. Some of us have already shown,[7b,9] that activators of CA I are similar to those of CA II, although differences were detected between the two isozymes.

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