Carbonic Anhydrase Activators. XV. A Kinetic Study of the Interaction of Bovine Isozyme II with Pyrazoles, Bis- and Tris-azolyl-methanes

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A series of eighteen substituted pyrazoles, bis- and tris-azolyl-methanes or ethanes was investigated for their interaction with the zinc enzyme carbonic anhydrase (CA). Several types of activities were detected, generally as CA activators, but CA inhibitory properties were also discovered for the very sterically-demanding derivatives of these series. Kinetic determinations by a stopped-flow technique for carbon dioxide hydration reaction allowed the determination of Michaelis–Menten constants, which are identical in the absence or in the presence of these modulators, proving a noncompetitive mechanism of activation–inhibition. MNDO calculations were used with moderate success to explain the biological results.

Key words carbonic anhydrase; isozyme II; azole; bis-/tris-azolyl-methane/-ethane; molecular modelling

Several classes of activators of the zinc enzyme carbonic anhydrase (CA, EC 4.2.1.1) have been reported,[2,3] including heterocyclic compounds such as imidazole, benzimidazole and pyrazole derivatives[2-5]; amines such as histamine, serotonin and diverse catecholamines[2,6-8]; amino acids and some of their derivatives[9-11] as well as medium-sized peptides.[9,12] All these compounds act by shuttling protons between the active site of the enzyme and its environment, and facilitate catalysis *via* enzyme–activator complexes, both for carbon dioxide hydration as well as ester hydrolysis reactions, as proved by us earlier.[4,5,10]

In a previous paper[5] we detected very strong CA activatory properties for azoles (imidazole and pyrazole derivatives), as well as for bis-azolyl-methanes and ethanes possessing a pK_a value in the range of 6.5—8.0 pK_a units. A quantitative relationship was also obtained between the activatory power and pK_a of the activator molecule, which together with the quantitative structure–activity relationship (QSAR) study recently reported by Clare and Supuran[10] constitute the only theoretical approaches to CA activation (in contrast to CA inhibition, which has been thoroughly investigated, also by means of theoretical calculations).[14,15]

Here we extend the previous work to other azole derivatives, also reporting a kinetic study of their interaction with bovine isozyme II. This brings novel insights regarding the mechanisms of CA activation with this type of compounds.

MATERIALS AND METHODS

Compounds **1**—**3** used in the enzymatic assays reported in this paper are either commercial (**1a**, **1d**, **1e**), or were as previously reported.[16] Bovine CA II and buffers were from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Kinetic Measurements Initial rates of carbon dioxide hydration were measured at 23 °C in a Hi-Tech stopped-flow apparatus by the changing-pH colorimetric method of Khalifah.[17] The buffer-indicator system was HEPES (5 mM)–4-nitrophenol, monitored at 400 nm, the enzyme concentration was 1.7×10^{-9} M, and the ionic strength was maintained at 0.2 M by the addition of sodium sulfate (a non-inhibiting anion for CA II).[14] The photomultiplier signal obtained was fed into a Multimixing Stopped-Flow SHU instrument, interfaced with an IBM-compatible PC possessing a DAS-50 Omega interface.

Initial CO_2 concentrations were determined by an enzymatic assay using the phosphoenol pyruvate carboxylase–malate dehydrogenase method[18] with a kit from Gilford Systems (Oberlin, OH, U.S.A.), spectrophotometrically, working at 340 nm, with a Gilford Response TM instrument. Rate data were fitted to the Michaelis–Menten equation (using Eadie–Hofstee plots) using a program written by us. Data acquisition was done using the RKBIN-1 program from Hi-Tech.

RESULTS AND DISCUSSION

The three simple pyrazoles **1a**—**c** previously investigated by us as CA activators,[5] were either totally devoid of such properties (**1a**, **b** with pK_a values around 2.0—2.5),[5] or weak activators (**1c**, with the pK_a of 3.74).[5] Mention should be made that although the enzymatic assay method used in the present study[17] is different from the one used previously (the Maren's method,[19] also a changing–pH colorimetric method), data obtained for the investigated compounds by these two assay methods (see Table 1) are in very good agreement.

The other derivatives, **1d**—**h** were not investigated previously, and have been included in the present study due to their substitution pattern: the presence of amino groups (such as in **1e**), which can possibly participate in additional proton transfer processes; the presence of a very bulky moiety (as in **1f**, possessing the adamantyl group), or, the presence of hydroxymethyl moieties (as in **1g**, **h**), which, in addition to influencing the pK_a of the respective compound, can eventually participate in supplementary hydrogen bonds with amino acid active site residues.

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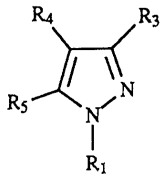
Table 1. Bovine CA II Activation with Pyrazoles **1**, at 10^{-5} M Concentration of Activator



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Compd.	R_1	R_3	R_4	R_5	%CA activation[a]
1a	H	H	H	H	100±3.9[b] 100±1.8[c]
1b	Me	H	H	H	100±3.9[b] 100±2.1[c]
1c	Me	Me	H	Me	118±4.1[b] 120±3.7[c]
1d	H	Me	H	Me	126±3.2[c]
1e	H	NH_2	H	H	112±1.5[c]
1f	H	H	Ad[d]	H	60±2.5[c,e]
1g	CH_2OH	H	H	H	156±3.5[c]
1h	CH_2OH	Me	H	Me	178±2.6[c]

a) Control CA activity in the absence of activator is (100±2.5)% (from 15 determinations); for all other data the values correspond to 5 determinations. *b*) From ref. 5). *c*) This study. *d*) Ad = 1-adamantyl. *e*) The compound is a CA inhibitor with an IC_{50} value of 1.3×10^{-5} M, and it acts as a non-competitive inhibitor with the substrate CO_2.

From the data of Table 1, the following features can be seen: (i) compounds **1d, e** act as weak CA activators, similarly to **1c** previously investigated. It is thus clear that the amino group in **1e** is not able to shuttle protons in the pH domain of interest for CA activation processes; (ii) a surprising finding was the fact that **1f** is a CA inhibitor, being the first pyrazole with such a behavior. In this context it is interesting to note the behavior of diverse simple azoles towards different CA isozymes: imidazole is an inhibitor of CA I,[17,20,21] as determined by X-ray crystallography[20] and spectroscopic methods,[21] but strongly activates isozyme CA II.[4,5] Other azoles, such as 1,2,4-triazole and tetrazole, were also reported to act as inhibitors for isozyme II,[22] and the crystal structure of human CA II with 1,2,4-triazole was recently reported.[23,24] Thus, it is probable that the very bulky adamantyl group in **1f** hinders the access of the compound within the active site, and as a consequence, it is unable to participate in proton transfer processes. Moreover, the complex formed between **1f** and the enzyme is strong enough to affect the catalysis of this very efficient isozyme (an IC_{50} of 1.3×10^{-5} M for **1f** was determined; IC_{50} represents the molarity of the inhibitor producing a 50% decrease of enzyme specific activity). Typically, sulfonamides, the prototypical CA inhibitors, have IC_{50} of the order of magnitude of 1—10 nM[15,25]; (iii) another surprising finding was the relatively strong activatory power of derivatives, **1g, h**, possessing l-hydroxymethyl moieties (see Table 1). Up to now, pyrazoles were considered to be very ineffective CA activators, mainly due to their low pK_a values (in the range of −2.1—3.4 pK_a units, see discussion in Ref. 5). Since the most effective pyrazole activator previously studied was **1c**[5] as seen from data of Table 1 as well as Fig. 1, compounds such as **1g, h** may act as quite potent activators (for instance at 0.1 mM, **1g** activates 228%).

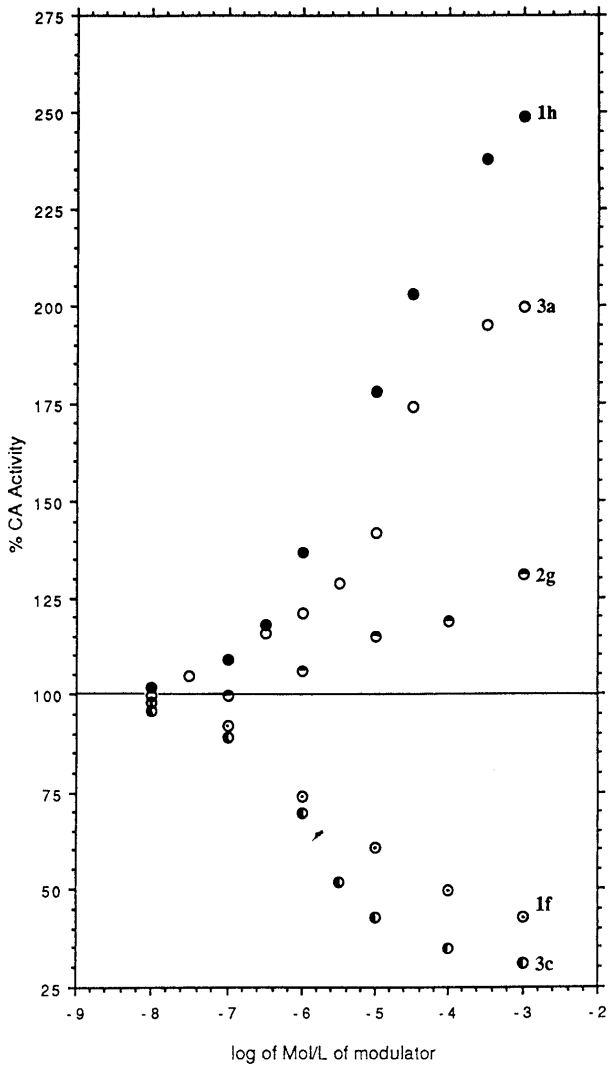
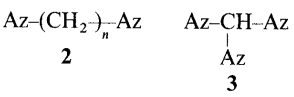


Fig. 1. Dose–Response Curves for the Interaction of CA II with **1**—**3**
Activation with **1h** ●, **3a** ○ and **2g** ◓; inhibition with **1f** ⊙ and **3c** ◐.

In the previous study[5] we also investigated bis-azolylmethanes and ethanes, Az-$(CH_2)_n$-Az ($n = 1, 2$) of type **2** (Az = (substituted)imidazole, pyrazole or benzimidazole). Such compounds possessed very strong CA activatory properties, when at least one pK_a value of these difunctional acids was in the range 6.4—8.0.[5] Among these derivatives, bis-imidazolylmethane **2a** is a moderately active compound, whereas the bis-imidazolylethanes **2b, c** are much stronger activators (see Table 2, where activities obtained by the two previously mentioned methods[17,19] are shown). Mention should be made that bis-pyrazolylmethane **2d**, as well as bis-pyrazolylethane **2e**, are totally ineffective as CA activators.

Thus, in order to gain new insights regarding the mechanisms of action of this class of compounds, as well as factors governing structure–activity relationships, some new derivatives of type **2** were included in this study (such as **2f, g**), together with tris-azolylmethanes of type **3** (Table 2). More than that, all these derivatives were also investigated kinetically, it having been discovered that they behave as non-competitive activators/inhibitors

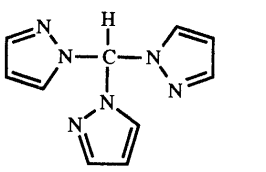
Table 2. Bovine CA II Activation with Derivatives **2** and **3**, at 10^{-5} M Concentration of the Compound



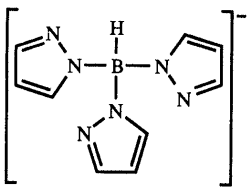
2 **3**

Compd.	AzH	*n*	%CA activation[a]
2a	1*H*-Imidazole	1	140±4.5[b]
			137±1.8[c]
2b	1*H*-Imidazole	2	154±4.8[b]
			161±3.7[c]
2c	2-Methyl-1*H*-imidazole	2	231±6.3[b]
			240±2.4[c]
2d	1*H*-Pyrazole	1	100±3.1 (4.2)[b,c]
2e		2	100±3.5 (3.3)[b,c]
2f	3-Amino-1*H*-pyrazole	1	129±1.4[c]
2g	1*H*-1,2,4-Triazole	1	115±2.1[c]
3a	1*H*-Pyrazole	—	142±2.5[c]
3b	3,5-Dimethyl-1*H*-pyrazole	—	153±1.0[c]
3c	1*H*-Benzimidazole	—	43±0.7[c,d]

a—*c*) Same as in Table 1. *d*) CA inhibitor, IC_{50} 10^{-5} M.



3a



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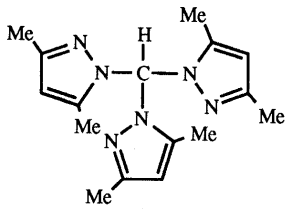
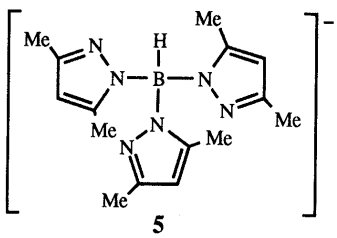


3b



5

with the substrate CO_2 (see later in the text).

From the data of Table 2 it can be seen that the bis-pyrazolylmethane **2f** behaves as a relatively weak CA activator, but it still possesses some activity as compared to the completely inactive unsubstituted derivative **2d**. Probably, the activity of **2f** may be accounted for by the shuttling capacity of the two amino groups present in its molecule. It should also be mentioned that **2f** is a stronger activator than **1e** (Table 1) which contains only one heterocyclic ring in its molecule, and only one NH_2 moiety. On the other hand, bis-triazolylmethane **2g** is a very weak activator, too (but in contrast to 1,2,4-triazole itself, which, as we mentioned, is a CA inhibitor.[22])

However, the most interesting derivatives from this series are probably the tris-azolylmethanes **3a**—**c**. These compounds are strikingly structurally similar to poly-(azolyl)borates of the type $HBAz_3$, as for instance the scorpionates $HBpz_3$ (**4**) or $HBdmpz_3$ (**5**), which share a common coordination chemistry.[26] Mention should be made that tetrahedral Zn(II) complexes of ligands such as **4**, **5** reversibly react with carbon dioxide, mimicking quite well the actual enzyme, CA.[27] In this context, it is

Table 3. Kinetic Data for Carbon Dioxide Hydration Reaction Catalyzed by CA II (1.7×10^{-9} M), at 23 °C (pH 7.5, 5 mM HEPES Buffer) and in the Presence of a 10^{-5} M Concentration of **1**—**3**

System	K_m (mM)[a]	V_{max} (mM l^{-1} s^{-1})[a]
CA II	9.4±0.1	6.24±0.11
CA II+**1c**	9.4±0.1	6.31±0.10
CA II+**1d**	9.3±0.2	6.33±0.09
CA II+**1f**	9.5±0.1	5.87±0.13
CA II+**1h**	9.3±0.2	6.66±0.10
CA II+**2a**	9.5±0.3	6.44±0.08
CA II+**2c**	9.4±0.2	6.63±0.15
CA II+**3a**	9.4±0.3	6.44±0.06
CA II+**3b**	9.5±0.3	6.52±0.11
CA II+**3c**	9.4±0.2	5.85±0.15

a) Mean±S.E. from 5 determinations.

interesting to note the good CA activatory properties of **3a**, **b** and the inhibitory properties of **3c**.

A comparison of dose-response CA activatory/inhibitory properties for some of the discussed compounds (**1f** and **3c** as inhibitors, and **1h**, **2g**, **3a** as activators) is detailed in Fig. 1. For all types of activities [weak (**2g**), moderate (**3a**) or strong (**1h**) activators, as well as for the two inhibitors] the effect is enhanced at increasing concentrations of the compound used in the assays (experiments with all derivatives discussed were done in the concentration range of 0.5 mM—10 nM).

Some kinetic data for the CA catalyzed hydration of carbon dioxide, with and without diverse derivatives **1**—**3**, are shown in Table 3 and Fig. 2 (obtained by the stopped flow method of Khalifah).[17]

From the above data it is obvious that activators/inhibitors from this class of compounds are of a non-competitive type with this substrate, since the Michaelis–Menten constants are practically identical in the presence/absence of the modulators. This is also seen in Fig. 2, where the Eadie–Hofstee plots are used, exemplifying the two activators (**1h**, **2c**) and two inhibitors (**1f**, **3c**) detected.

These data allow us to draw the following conclusions. The mechanism of action of these modulators of CA activity is of a non-competitive type, implying that in the ternary complex enzyme-activator-CO_2, the substrate and the activator are bound in different regions of the active site, but presumably not very far from each other, so that the activator should be able to shuttle protons outside the active site and facilitate the formation of the nucleophilic (active) species of the enzyme (with a hydroxide ion bound to Zn(II)). For inhibitors (the first time detected in the pyrazole series), binding within the active site impairs catalysis, probably by steric effects, since the two inhibitors detected in this series are both very bulky (one containing an adamantyl moiety, and the other the tris-benzimidazolylmethane system). Thus, we proved that in addition to the pK_a of the modulator molecule, which is a critical parameter both for CA inhibition[3,4] as well as CA activation,[5,10] the structural factor also plays a very important role. Practically, a relatively small structural variation, such as the presence of a bulky group in such a heterocyclic derivative, not only completely abolished CA activatory properties, but led to compounds possessing CA inhibitory properties as well. Considering

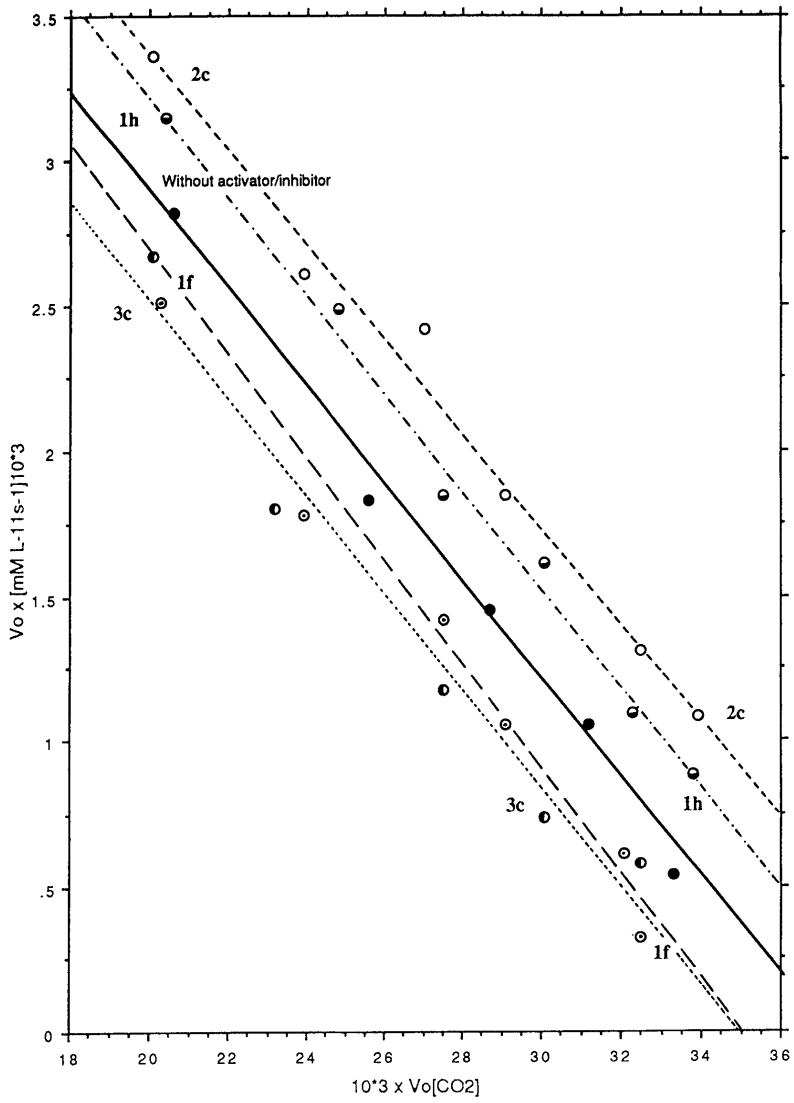


Fig. 2. Eadie–Hofstee Plots for CA II (1.7×10^{-8} M, 23 °C, 5 nM HEPES buffer, pH 7.5) and in the Presence of 10^{-5} M of **1f**, **2c** and **3c**
Compounds: **2c** ○, **1h** ◒, **1f** ◐, **3c** ⊙. Without activator/inhibitor ●.

the complex stereoelectronic factors that govern biological activity, the present results might be useful for designing stronger enzyme activators/inhibitors.

Semiempirical Calculations We have tried to rationalize and, if possible, to explain quantitatively the biological results. With this purpose in mind we have carried out semi-empirical calculations. After several attempts (AM1, PM3), we found that the best results were obtained using Dewar's MNDO Hamiltonian. We have collected data corresponding to seventeen compounds of the present publication (4-adamantylpyrazole **1f** was not calculated) and nine of the previous one[5] in Table 4.

We have already used log ratio in the preceding paper [ratio $=(\%A_{max}-\%A_i)/(\%A_i-\%A_{min})$, $\%A_{max}=225$ and $\%A_{min}=109$]. The pK_a values are from two sources, one for simple compounds[28] and the other for the first protonation of bis- and tris-azolylalkanes.[29] All the other data came from MNDO calculations within the MOPAC 6.0 package (in all cases the geometries were fully optimized).[30]

The calculations involve the 26 neutral molecules and the corresponding 26 monoprotonated cations. Only in the case of bis-1,2,4-triazolylmethane **2g** is there an ambiguity concerning the protonation site; we have calculated the cation corresponding to the monoprotonation on N4.[28] The Δ_{qN} is the difference in the charge of the most basic nitrogen (N2 in pyrazoles, N3 in imidazoles and benzimidazoles and N4 in triazole **2g**) between the neutral molecule and the cation, that is, the effect of the protonation on the total charge. These calculations also provided the HOMO and LUMO energies (in eV). Finally, the log P values were calculated using the MEDCHEM program (version 3.54).[31]

Amongst the many correlations we tried, the only really interesting are:

$$pK_a \text{ (imidazoles, benzimidazoles, triazoles)} = 12.6 \pm 0.2 - 100 \pm 3\ \Delta_{qN}$$
$$n=10, \quad r^2=0.994 \qquad (1)$$

$$pK_a \text{ (pyrazoles)} = -190 \pm 17\ \Delta_{qN}$$
$$n=9, \quad r^2=0.940 \qquad (2)$$

$$pK_a \text{ (all)} = 100 \pm 10 + 10.2 \pm 1.1 \text{ LUMO}$$
$$n=19, \quad r^2=0.828 \qquad (3)$$

Table 4. MNDO Calculated Properties of CA Activators from Compounds of This and the Preceding Publication[5)]

	No. (This work)	No. (Ref. 5)	Name	log ratio	pK_a	Δ_{qN}	HOMO	LUMO	log P
1	**1a**	**5a**	1*H*-Pyrazole	—	2.50	−0.009	−9.903	−9.614	0.321
2	**1b**	**5b**	1-Methylpz	—	2.06	−0.010	−9.744	−9.531	0.647
3	**1c**	**5c**	1,3,5-Trimethylpz	1.13	3.74	−0.024	−9.543	−9.446	1.945
4	**1d**	—	1*H*-3,5-Dmpz	0.88	4.06	−0.018	−9.690	−9.527	1.619
5	**1e**	—	1*H*-3(5)-Aminopz	1.68	—	−0.045	−9.672	−9.085	−0.906
6	**1g**	—	1-Hydroxymethylpz	0.32	—	0.019	−9.884	−9.584	−0.063
7	**1h**	—	1-Hydroxymethylpz-dmpz	0.05	—	0.009	−9.675	−9.476	1.235
8	**2a**	**6a**	Bis-im-methane	0.61	5.56	0.069	−9.368	−9.354	−0.326
9	**2b**	**6c**	Bis-im-ethane	0.30	6.41	0.065	−9.333	−9.302	−0.144
10	**2c**	**6d**	Bis-mim-ethane	−0.82	7.28	0.054	−9.235	−9.205	1.154
11	**2d**	**8a**	Bis-pz-methane	—	0.12	0.001	−9.690	−9.667	0.903
12	**2e**	**8b**	Bis-pz-ethane	—	1.67	−0.003	−9.716	−9.574	0.990
13	**2f**	—	Bis-3-aminopz-methane	0.80	—	−0.047	−9.288	−9.137	−0.687
14	**2g**	—	Bis-triazolylmethane	1.37	0.11	0.124	−10.513	−9.685	−1.883
15	**3a**	—	Tris-pz-methane	0.54	—	−0.002	−9.772	−9.591	1.771
16	**3b**	—	Tris-dmpz-methane	0.36	—	−0.017	−9.551	−9.478	5.665
17	**3c**	—	Tris-benzimidazolyl-methane	—	—	0.094	−9.226	−9.194	4.440
18	—	**4a**	1*H*-Imidazole	−0.10	6.99	0.055	−10.714	−9.083	−0.047
19	—	**4b**	1-Methylim	−0.14	7.12	0.054	−10.489	−9.047	0.122
20	—	**4c**	1-Ethylim	−0.26	7.19	0.053	−10.450	−9.016	0.651
21	—	**4d**	1,2-Dimethylim	−1.24	8.00	0.045	−10.464	−8.960	0.771
22	—	**6b**	Bis-mim-methane	0.16	6.64	0.056	−9.232	−9.205	1.154
23	—	**7**	Bis-benzimidazolyl-ethane	1.37	4.61	0.079	−9.087	−9.050	3.044
24	—	**8c**	Bis-dmpz-methane	—	2.14	−0.011	−9.526	−9.511	3.499
25	—	**8d**	Bis-dmpz-ethane	1.68	3.39	−0.018	−9.577	−9.474	3.586
26	—	**8e**	Bis-4-bromopz-ethane	—	0.00	−0.004	−9.809	−9.697	2.716

pz = pyrazole, dmpz = 3,5-dimethylpyrazole, im = imidazole, mim = 2-methylimidazole.

$$\log \text{ratio} = 2.1 \pm 0.3 - 0.35 \pm 0.05\ pK_a + 0.19 \pm 0.08 \log P$$
$$n = 13, \quad r^2 = 0.825 \qquad (4)$$

It is known that relationships between the basicity of azoles and theoretical properties such as charge density, results in two equations, one for pyrazoles and the other for the remaining azoles.[28)] In our case, this corresponds to Eqs. 1 and 2; using the LUMO values, all compounds fit in one line, Eq. 3, but of low quality; this regression is not good enough to be used for predicting the missing pK_a.

Equation 4 corresponds to all the active compounds (19) for which the corresponding pK_a are known (only 13). Equation 4 does not apply to the six compounds (**1e**, **1g**, **1h**, **2f**, **3a**, **3b**) whose pK_a are not known, especially aminopyrazoles **1e** and **2f** and 1-hydroxy-methylpyrazoles **1g** and **1h**, which have other functionalities in the molecule.

To have powerful CA activators, the pK_a should be large and positive (imidazoles), since the slope is negative, and the log P should be large and negative since the slope is positive. An analysis of log P in terms of different contributions lead to Eq. 5:

$$\log P = 0.6 \pm 0.1 \text{ (number of pyrazoles)} - 0.2 \pm 01 \text{ (number of imidazoles)} - 1.0 \pm 0.2 \text{ (number of triazoles)} + 1.4 \pm 0.1 \text{ (number of benzimidazoles)} - 0.4 \pm 0.2 \text{ (number of NH or OH)} + 0.2 \pm 0.1 \text{ (number of } N\text{-CH, } N\text{-CH}_2 \text{ or } N\text{-CH}_3) + 0.6 \pm 0.1 \text{ (number of C-CH}_3)$$
$$n = 27, \quad r^2 = 0.974 \qquad (5)$$

Thus, to have negative log P (hydrophobic compounds) *N*-methyl, *N*-methylene, C-methyl and benzo groups must be avoided. In conclusion, MNDO and log P calculations provide at best a partial answer to our attempt to use QSAR in the case of CA activators.

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