# Carbonic Anhydrase Activators: Design of High Affinity Isozymes I, II, and IV Activators, Incorporating Tri-/Tetrasubstituted-pyridinium-azole Moieties

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A series of tight binding carbonic anhydrase (CA) activators was obtained by reaction of amino-azoles (3-amino-pyrazole, 2-amino-imidazole, and 5-amino-tetrazole) with tri- or tetrasubstituted pyrylium salts. Many of the new pyridinium salts incorporating azole moieties reported here proved to be efficient in vitro activators of three CA isozymes, CA I, II, and IV. Very good activity was detected against hCA I and bCA IV (h = human; b = bovine isozymes), for which some of the new compounds showed affinities in the low nanomolar range, whereas against hCA II, their affinities were in the range of 95−150 nM. Substitution patterns of the pyridinium ring leading to best activity included 4-phenyl-2,6-dialkyl moieties or 2,4,6-tri- and 2,3,4,6-tetraalkyl groups. Ex vivo experiments showed some of the new activators to strongly enhance CA activity after incubation with human erythrocytes. Furthermore, due to their cationic nature, some of these compounds (the imidazole and pyrazole derivatives) are membrane-impermeant, discriminating thus between cytosolic and membrane-bound CA isozymes. The present paper is the first report of membrane-impermeant CA activators. The pyridinium tetrazole derivatives on the other hand do penetrate through biological membranes. Such CA activators might lead to the development of drugs/diagnostic tools for the management of CA deficiency syndromes as well as for the pharmacological enhancement of synaptic efficacy, spatial learning, and memory. This may constitute a new approach for the treatment of Alzheimer disease and other conditions in need of achieving memory therapy.

## Introduction

Activation of the zinc enzyme carbonic anhydrase (CA, EC 4.2.1.1) by a multitude of physiologically relevant compounds such as biogenic amines (histamine, serotonin, catecholamines), amino acids, or oligopeptides/small proteins among others, has only recently been explained at the molecular level.[1−3] By means of electronic spectroscopy, X-ray crystallography, and kinetic measurements, it has been proved that the activator molecule binds within the enzyme active cavity at a site distinct of the inhibitor or substrate binding sites, participating thereafter in the rate-determining step of the catalytic cycle, i.e., the proton-transfer processes between the active site and the environment.[1−3]

Except for clarifying basic aspects of the catalytic mechanism of this class of widely spread enzymes over the phylogenetic tree,[4,5] CA activators might also possess pharmacological applications, although this field is largely unexplored for the moment. Thus, very recently it has been reported[6] that phenylalanine, a CA activator first investigated by this group,[1,3] when administered to experimental animals produces an im-

portant pharmacological enhancement of synaptic efficacy, spatial learning, and memory, proving that this class of unexplored enzyme modulators may be used for the management of conditions in which learning and memory are impaired, such as Alzheimer's disease or aging. It should be also mentioned that it was previously reported that the levels of several CA isozymes are significantly diminished in the brain of patients affected by Alzheimer's disease,[7] a fact strongly supporting the involvement of CAs in cognitive functions.[4,6,7]

In previous contributions from this laboratory[9−13] it was shown that effective CA activators can be designed by considering histamine **1** as a lead molecule.[2] Indeed, the X-ray crystallographic structure of the adduct of human CA II (hCA II) with histamine, a moderate-weak activator (activation constant, $K_A$ = 125 $\mu$M), showed the activator molecule to be bound at the entrance of the active site cavity, where it is anchored by hydrogen bonds to three amino acid side chains and to a water molecule. These hydrogen bonds involve only the nitro-
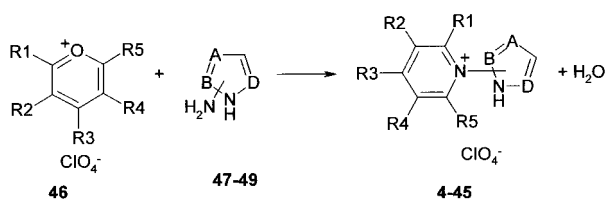
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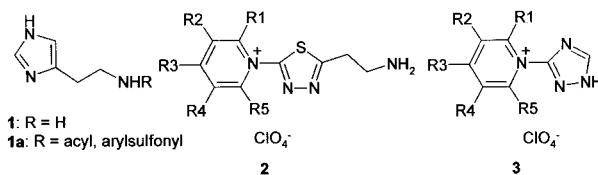
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**1**: R = H
**1a**: R = acyl, arylsulfonyl
**2**
**3**

**Scheme 1**[a]



[a] A, B, D = N or C.

gen atoms of the imidazole moiety of histamine (the $N\delta1$ and $N\epsilon2$ are engaged in hydrogen bonds with the side chains of Asn 62, His 64, Gln 92, and with Wat 152, respectively), whereas the aliphatic amino group is not experiencing any contact with the enzyme but is extending away from the cavity into the solvent.[2] Positioned in such a favorable way, histamine facilitates the rate-limiting step of CA catalysis but also allows its easy derivatization (at the aliphatic amino group) in order to obtain tighter-binding activators, as it has been reported by us for compounds incorporating acyl or arylsulfonyl moieties, of type **1a**, which behaved as much stronger CA activators against isozymes CA I, II, and IV, as compared to the lead **1**.[8-10]

Again considering histamine as lead, this group reported[12] the good CA activatory properties of a series of aminoethyl-1,3,4-thiadiazoles of type **2**, also incorporating substituted pyridinium moieties in their molecule. A further increase of the CA activatory properties was then found for a series of 1,2,4-triazoles incorporating substituted pyridinium moieties, of type **3**.[13] It became thus clear that effective CA activators should incorporate two main structural elements: (i) a moiety able to participate in proton-transfer reactions between the active site and the bulk solvent, preferably with a $pK_a$ in the range of 6−8;[1,11,14] (ii) additional moieties that ensure a tight binding to the enzyme, of the arysulfonamido,[8] acylamido,[9] or substituted pyridinium[12,13] type. Considering the excellent CA activatory properties reported in a preliminary communication[13] for the pyridinium-triazoles **3**, we decided to investigate in more detail this type of derivative. Here we report the synthesis and CA activation of three physiological relevant isozymes (CA I, II, and IV) with three series of novel 1-azolyl-pyridinium derivatives incorporating pyrazole, imidazole, and tetrazole, respectively, as proton shuttle moieties between the CA active site and the environment. A comparative CA in vitro and ex vivo activation study was performed, and some elements of structure−activity relationship in this class of CA activators are also discussed.

## Results

**Synthesis.** Preparation of the new compounds **4−45** is shown in Scheme 1 and involved the reaction of tri-/tetrasubstituted pyrylium salts[15] **46** with the amino azoles **47−49**.[15-18] All new compounds were characterized by standard analytical and spectroscopic methods that confirmed the proposed structures.

**Carbonic Anhydrase Activation.** In vitro activation data of isozymes hCA I, hCA II, and bCA IV with the new derivatives **4−45**, as well as standard activators, is presented in Table 1.

**Table 1.** CA Activatory Properties against Isozymes CA I, II, and IV of Compounds **4−45** Prepared in the Present Study, Compared with Those of Histamine as Standard



| compd | R1 | R2 | R3 | R4 | R5 | hCA I[b] $K_A(\mu M)$[a] | hCA II[c] $K_A(\mu M)$[a] | bCA IV[d] $K_A(\mu M)$[a] |
|---|---|---|---|---|---|---|---|---|
| **1** (histamine) | | | | | | 2.10 | 125 | 41 |
| **4** | Me | H | Me | H | Me | 0.052 | 0.480 | 0.045 |
| **5** | Me | H | Me | Me | Me | 0.049 | 0.457 | 0.038 |
| **6** | Me; R2,R4 = (CH2)9, R3 = H | | | | Me | 0.037 | 0.412 | 0.033 |
| **7** | Me | H | Me | H | Ph | 0.875 | 83 | 1.68 |
| **8** | Me | H | Ph | H | Me | 0.035 | 0.359 | 0.017 |
| **9** | Et | H | Ph | H | Et | 0.027 | 0.306 | 0.015 |
| **10** | *n*-Pr | H | Ph | H | *n*-Pr | 1.35 | 75 | 1.20 |
| **11** | *i*-Pr | H | Ph | H | *i*-Pr | 0.021 | 0.155 | 0.008 |
| **12** | *n*-Bu | H | Ph | H | *n*-Bu | 1.76 | 140 | 2.59 |
| **13** | Me | H | Ph | H | Ph | 0.64 | 96 | 1.85 |
| **14** | Et | H | Ph | H | Ph | 2.34 | 118 | 3.41 |
| **15** | *n*-Pr | H | Ph | H | Ph | 2.78 | 135 | 8.23 |
| **16** | *i*-Pr | H | Ph | H | Ph | 2.50 | 130 | 5.29 |
| **17** | *n*-Bu | H | Ph | H | Ph | 5.64 | >150 | >50 |
| **18** | Ph | H | Ph | H | Ph | 32 | >150 | >50 |
| **19** | *i*-Pr | H | Me | H | *i*-Pr | 0.038 | 0.264 | 0.016 |
| **20** | Me | H | Me | H | Me | 0.036 | 0.347 | 0.021 |
| **21** | Me; R2,R4 = (CH2)9, R3 = H | | | | Me | 0.022 | 0.305 | 0.020 |
| **22** | Me | H | Me | H | Ph | 0.360 | 59 | 0.975 |
| **23** | Me | H | Ph | H | Me | 0.021 | 0.153 | 0.013 |
| **24** | Et | H | Ph | H | Et | 0.018 | 0.144 | 0.009 |
| **25** | *n*-Pr | H | Ph | H | *n*-Pr | 0.247 | 48 | 0.620 |
| **26** | *i*-Pr | H | Ph | H | *i*-Pr | 0.012 | 0.095 | 0.006 |
| **27** | *n*-Bu | H | Ph | H | *n*-Bu | 0.651 | 86 | 1.89 |
| **28** | Me | H | Ph | H | Ph | 0.303 | 62 | 1.24 |
| **29** | Et | H | Ph | H | Ph | 1.05 | 84 | 2.56 |
| **30** | *n*-Pr | H | Ph | H | Ph | 1.90 | 130 | 5.76 |
| **31** | *i*-Pr | H | Ph | H | Ph | 1.54 | 107 | 3.14 |
| **32** | *n*-Bu | H | Ph | H | Ph | 2.40 | 130 | 9.55 |
| **33** | Ph | H | Ph | H | Ph | 15 | >150 | >50 |
| **34** | *i*-Pr | H | Me | H | *i*-Pr | 0.024 | 0.130 | 0.008 |
| **35** | Me | H | Ph | H | Me | 0.076 | 1.39 | 0.063 |
| **36** | Et | H | Ph | H | Et | 0.064 | 1.23 | 0.055 |
| **37** | *n*-Pr | H | Ph | H | *n*-Pr | 2.12 | 136 | 1.55 |
| **38** | *i*-Pr | H | Ph | H | *i*-Pr | 0.050 | 1.15 | 0.012 |
| **39** | *n*-Bu | H | Ph | H | *n*-Bu | 1.80 | 145 | 6.80 |
| **40** | Me | H | Ph | H | Ph | 1.10 | 108 | 5.24 |
| **41** | Et | H | Ph | H | Ph | 3.05 | 140 | 12.52 |
| **42** | *n*-Pr | H | Ph | H | Ph | 4.21 | >150 | 15.50 |
| **43** | *i*-Pr | H | Ph | H | Ph | 2.87 | 134 | 10.13 |
| **44** | *n*-Bu | H | Ph | H | Ph | 10.50 | >150 | >50 |
| **45** | Ph | H | Ph | H | Ph | 41 | >150 | >50 |

[a] Activation constant, mean from at least three determinations by the esterase method. Standard error was in the range of 5−10%. [b] [hCA I] = 25 nM. [c] [hCA II] = 7 nM. [d] [bCA IV] = 33 nM.

**Ex Vivo Activation.** Data with some of the best in vitro activators, against human red cell isozymes (hCA I + hCA II), after incubation of red cells with the activator solution for different periods of time, are presented in Table 2.

## Discussion

**Chemistry.** A series of 2,4,6-tri-/2,3,4,6-tetrasubstituted pyrylium salts were condensed with 3-amino-pyrazole **47**, 2-amino-imidazole **48**, and 5-amino-tetrazole **49** in Baeyer−Piccard reaction conditions, affording

**Table 2.** Ex Vivo CA Activation Data after 30 and 60 min of Incubation of Lysed Human Erythrocytes with Solutions Containing 5 $\mu$M Activators: Histamine (as standard), **11**, **23**, **26**, and **38**

| activator | % CA activity[a] | |
|---|---|---|
| | 30 min | 60 min |
| histamine | $121 \pm 3$ | $130 \pm 5$ |
| **11** | $189 \pm 5$ | $234 \pm 9$ |
| **23** | $192 \pm 8$ | $233 \pm 11$ |
| **26** | $256 \pm 7$ | $298 \pm 10$ |
| **38** | $173 \pm 6$ | $212 \pm 8$ |

[a] Mean $\pm$ standard error ($n = 3$); erythrocyte CA activity (hCA I + hCA II) in the absence of activator is taken as 100%.

**Scheme 2**



**54**

**53**

(For R6 or R2 = Me or CH₂R')
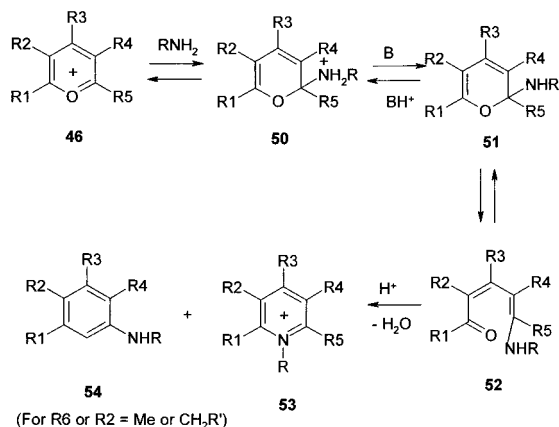
three series of new pyridinium salts: **4−19**, **20−34**, **35−45**, respectively (Scheme 1). Although apparently very simple, the reaction is in reality a multistep process (Scheme 2), the key factors influencing it being the azole amino group nucleophilicity, the pyrylium salt reactivity, the steric hindrance of the heterocyclic ring, and possible side reactions, as shown previously by these and other researchers.[13,15,16,19−23] Thus, the nucleophilic attack of a primary amine RNH$_2$ on pyrylium cations generally occurs in the α position, with the formation of intermediates of type **50**, which by deprotonation in the presence of bases lead to the 2-amino-tetradehydro-pyran derivatives **51**. In many cases the deprotonation reaction is promoted by the amine itself, when this is basic enough (this being the reason in many cases one works at the molar ratio pyrylium salt:amine of 1:2), or by external catalysts added to the reaction mixture, such as triethylamine.[15,16,19−22] The derivatives **51** are generally unstable, being tautomers with the ketodiene-amines **52**, which are the key intermediates for the conversion of pyryliums into pyridiniums.[15,16,19−22] In acidic media, in the rate-determining step of the whole process, ketodieneamines **52** may be converted to the corresponding pyridinium salts **53**, although other products such as vinylogous amides with diverse structures have also been isolated in such reactions.[15] A supplementary complication appears when the moiety substituting the 2- and/or 6-position(s) of the pyrylium ring is methyl (or CH$_2$R' and CHR'R''), in which case a concurrent cyclization with formation of the anilines **54** in addition to the pyridinium salts **53** may take place too.[15,16,19−22] These concurrent reactions mentioned above are generally important when the amine to be converted into the pyridinium salt possesses weak nucleophilicity or basicity. This probably happens to be

the case of some of the amines **47−49** investigated here, and consequently, despite laborious synthetic and purification procedures (see Experimental Section for details), yields in pyridinium salts **4−45** varied in a wide range (10−98%), being linked with the above-mentioned parameters.

Thus, especially for pyrylium salts possessing branched alkyl chains (e.g., *i*-propyl, *tert*-butyl, nonamethylene) in the 2- and/or 6-positions of the heterocyclic ring, the desired product could difficultly be obtained in pure form, due to the following problems: (i) preponderance of side reactions (mainly the above-mentioned C-cyclization, generating the corresponding substituted anilino-azoles of type **54**) and (ii) particularly high water solubility of the obtained products **53** and **54**. We were confronted with these problems during the preparation of 2,4,6-trimethyl- and 2,3,4,6-tetramethyl-pyridinium-tetrazole as well as 2,3,4,6-tetramethyl-pyridinium-imidazole (this situation has also been encountered at the preparation of pyridinium-1,2,4-triazoles[13] and pyridinium-benzenesulfonamides[22] reported previously by our group). Consequently, although TLC indicated the formation of these products, they were not included in the list of derivatives **4−45** shown in Table 1, due to their unsatisfactory purity.

For insight into the structure−activity relationship for this class of CA activators, we pursued some structural variations in the choice of the reactants, as follows: (i) the number of azole nitrogen atoms (2 or 4) and their position (pyrazole/imidazole), also for comparing these new activators with the previously mentioned analogues incorporating pyridinium-triazoles moieties, of type **3**;[13] (ii) the substitution pattern of the pyrylium ring (alkyl/aryl groups as well as different combinations of them), as this was one of the parameters that strongly influenced the efficacy of the pyridinium derivatives, both as CA activators[11,13] as well as CA inhibitors (for some pyridinium-sulfonamides reported previously).[22,23]

It must also be mentioned that the pyridinium tetrazoles **35−45** reported here were isolated as zwitterionic species (and not as perchlorates, as for the corresponding imidazoles and pyrazoles). This may be due to the very acidic character of this ring system (a typical isosteric replacement of the COOH moiety) and has also been observed for the pyridinium amino acid derivatives reported by Balaban's group.[15,16]

**In Vitro CA Activation.** The pyridinium-azoles **4−45** were assayed for activatory properties against isozymes hCA I, hCA II, and bCA IV, using histamine **1** as standard. Indeed, the new derivatives **4−45** generally showed good CA activatory properties. Activation data given in Table 1 reveal significant differences in isozyme specificity related to both "classical" activators such as histamine and the newly synthesized compounds. Thus, the most susceptible isozyme to activation by histamine was hCA I, followed at a rather large distance by bCA IV, whereas isozyme hCA II was shown to be less activated by this compound (more precisely, the difference in the three isozymes sensitivity expressed as activation constants is about 1 order of magnitude).[2] In the case of the pyridinio-azoles **4−45**, all three isozymes showed a 3-fold increased susceptibility to be activated by some of the new derivatives, as

compared to histamine. The isozymes affinity for the new activators was also different: although the cytosolic rapid isozyme hCA II remained the most reluctant to be activated (as for histamine), there were no significant differences between isozymes hCA I and bCA IV, which were both strongly activated by some of the compounds **4–45**. Regarding the intensity of the activation effect, the histamine pattern is maintained, the activation constants for hCA II being an order of magnitude higher than those for hCA I and bCA IV isozymes.

Regarding the previous study[13] on CA activation, the investigated isozyme affinity for the analogous series of pyridinium-1,2,4-triazoles varied in a different way, i.e., bCA IV ≫ hCA I > hCA II. It should be noted that, similarly to histamine, CA activation by the pyridinium-triazoles reported earlier[13] only led to micromolar activators, whereas many of the derivatives obtained in the present work showed nanomolar affinity to isozymes I and IV.

The influence of the pyridinium ring substitution pattern on the biological activity of compounds **4–45** confirmed and extended the conclusions of our preliminary study:[13] (i) derivatives possessing 2,6-dialkyl-4-phenyl moieties, such as **8–11**, **23–26**, and **35–38**, respectively, were the most active against all the investigated isozymes (with the 2,6-alkyl groups: *i*-Pr > Et > Me), confirming that elongation of the activator molecule in the direction of the axis passing through the Zn(II) ion of the enzyme, the activator molecule itself, and the exit of the active site is the most beneficial substitution pattern leading to strong CA activators; (ii) the 2,4,6-trimethyl- and 2,3,4,6-tetramethyl-pyridinium-azoles (**4**, **20,** and **5**) were slightly less active than the previously mentioned derivatives; (iii) the increased volume of the pyridinium ring moieties generated an opposite effect on the CA activation properties, with 2,4-diphenyl-6-alkyl-substituted derivatives (**13–17**, **28–32**, **40–44**, respectively) acting as moderate-weak activators [mention should be made that, as part of this pattern substitution, the most active derivative was the first term of the series, i.e., the compound bearing 2,4-diphenyl-6-methyl-pyridinium groups (**13**, **28**, **40**, respectively), with the higher homologues much less active (i.e., the 6-ethyl-, 6-propyl-, or 6-butyl derivatives)]; (iv) the 2,4,6-triphenyl-pyridinium azoles were practically devoid of activity, confirming the limited amount of space available in the activators' binding site;[1,13] and (v) the 2,6-dimethyl-3,5-nonamethylene-pyridinium azoles **6** and **21** (a substitution pattern newly introduced in this study) proved good in vitro CA activation properties. These lipophilic, flexible lariate side chain substituted derivatives showed indeed remarkable CA activatory properties, being only slightly less effective than the 2,6-dialkyl-4-phenyl-substituted azoles mentioned above.

Also in agreement with the structure–activity relationships data pointed out in our preliminary study,[13] we wish to stress here again that the alkyl chain length of the 2,6-substituents of the pyridinium ring is the most important factor determining the intensity of CA activation. Thus, considering the series of the 2,6-dialkyl-4-phenyl-substituted derivatives reported here, it was observed that passing from methyl to ethyl led to a slight increase in activity. On the contrary, the further

chain increase to *n*-propyl and *n*-butyl reduced the activatory properties, probably due to steric hindrance effects, which destabilize the activator interaction with the CA active site. The optimal fit has been achieved by 2,6-di-*i*-propyl-4-phenyl groups, with compounds **11**, **26**, and **38**, respectively, being the best activators in the whole series and against all three CA isozymes.

The nature of the azole ring is probably the second other important variable determining the activation effect in these series of compounds. Presuming an identical substitution pattern, the following order related to the activatory properties has been observed: imidazoles > pyrazoles ≫ tetrazoles. The explanation of this activity pattern may reside in the p$K_a$ values of the azolyl moieties in aqueous system: the imidazole ring system, with a p$K_a$ of 7.1 for proton acceptance (and 17.5 for H$^+$ donation), is the best option for shuttling protons between the active site and the external medium.[11,14] Pyrazole is much less basic than imidazole (p$K_a$ = 2.5 for proton acceptance), and this may explain the decreased CA activatory properties of its derivatives, as compared to the corresponding imidazoles.[11,14] Tetrazole, on the other hand, is a quite acidic compound (p$K_a$ = 4.9 for proton donation) and hence less appropriate to act as a CA activator (in neutral state). It should also be stressed that the p$K_a$ of the imidazole/pyrazole/tetrazole derivatives reported by us may be rather different from the p$K_a$ value of the parent ring systems, but this parameter should vary in the same manner for the three classes of derivatives investigated here and may be thus enlightening for better understanding SAR of such CA activators.

We also observed that the pyridinium-tetrazole derivatives reported here possess a zwitterionic nature (see structures **35–45**). Thus, it is rather probable that the pyridinium-imidazole/pyrazole derivatives reported in this paper bind to the enzyme as cations (and by means of their azole moieties shuttle protons between the Zn-bound water and the environment), whereas the pyridinium-tetrazoles prepared by us may bind as zwitterions, activating CAs by the same mechanism of action as the first type of compounds mentioned above, i.e., favoring the rate-determining proton-transfer reactions between the active site and the reaction medium. It may be mentioned again that the p$K_a$ of a compound bound within an enzyme active site may be quite different from the p$K_a$ of the same compound in solution.

**Ex Vivo CA Activation.** After incubation of normal blood red cells (containing approximately 150 $\mu$M of hCA I and 20 $\mu$M of hCA II)[24–26] with micromolar concentrations of histamine, the total CA activity (measured by the esterase method, with 4-nitrophenyl acetate as substrate)[25] in homogenates of treated cells is enhanced as compared to that of cells treated in a blank experiment only with buffer (Table 2). Performing the same type of experiment with some of the new activators synthesized in the present work (such as the pyrazole **11**, or the imidazoles **23** and **26**) does not lead to any CA activatory effects (data not shown). This is obviously due to the cationic nature of these derivatives, and in consequence, to their membrane impermeability, as already proved by us for positively charged sulfonamide CA inhibitors of type **55–58**.[22,23] Indeed, such positively

55: n = 0
56: n = 1
57: n = 2

58
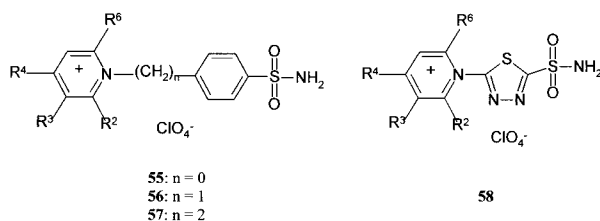
charged derivatives cannot cross the plasma membrane and are unable to arrive within the cytosol for activating CA I and II isozymes abundant in this cellular localization. To observe the ex vivo CA activatory properties of these pyridinium-azole derivatives, the experimental protocols described above had to be changed. Thus, red blood cells were first lysed in order to liberate the present CA isozymes, and then the lysate was incubated either with histamine or with the newly synthesized compounds **11**, **23**, or **26** (Table 2). After 30 or 60 min incubation, a clear-cut activatory effect was observed with the two types of compounds (histamine as standard, and the new positively charged activators reported here). Thus, histamine produced only a weak activation of around 120% after half an hour incubation, and of around 130% of the basal CA activity after 1 h incubation with red cells, either in the first as well as in the second type of experiment. Some of the new derivatives tested ex vivo (which showed strong in vitro CA activity enhancements) produced activations of around 190–255% after 30 min and 233–300% after 1 h incubation, respectively. The stronger in vitro CA activator **26** was also the most effective ex vivo derivative, as compared to the weaker activators **11** and **23** investigated in some detail. In contrast to the pyrazole and imidazole cationic derivatives mentioned above, the zwitterionic tetrazoles (such as **38**) do penetrate through the plasma membranes, since the ex vivo activatory effect of this compound is similar when incubating the blood red cells with the activator followed by lysis or when lysing first blood red cells and incubating them with the activator (Table 2). Thus, similarly to histamine and in contrast to the pyridinium-pyrazole/imidazole derivatives, the zwitterionic pyridinium tetrazoles do penetrate membranes activating thus cytosolic CA isozymes.

## Conclusions

Three series of 1-azolyl-tri-/tetrasubstituted pyridinium salts were synthesized by reaction of the corresponding pyrylium salts with 3-amino-pyrazole, 2-amino-imidazole, and 5-amino-tetrazole, respectively. Many of these new compounds proved to be powerful in vitro CA activators against isozymes I, II, and IV, with activities in the low nanomolar range for isozymes I and IV, and low micromolar range for isozyme II, showing thus a 3-fold increased activity as compared to histamine, the lead molecule used for their preparation. The different isozymes showed diverse affinities for these activators, with CA I ≈ CA IV > CA II. Furthermore, the best in vitro activators strongly enhanced ex vivo red cell lysate CA activity. SAR in these series was also discussed. These results confirmed and completed a previous CA activation study of our group on a series of 1-[1,2,4-triazole-(1*H*)-3-yl]-2,4,6-trisubstituted-pyridinium salts. Furthermore, this is the first report of membrane-impermeant CA activators.

## Experimental Section

**General.** Melting points were determined on a Boetius heating plate microscope and are uncorrected. The IR spectra were recorded in KBr pellets, on a Nicolet Avatar 360 FTIR spectrometer, in the range 400–4000 cm$^{-1}$. The NMR spectra were recorded in DMSO-$d_6$, at ≈295 K with a Varian Gemini 300BB spectrometer operating at 300 MHz for $^1$H and at 75 MHz for $^{13}$C. Chemical shifts are reported as $\delta$ values, using TMS as internal standard for proton spectra and the solvent resonance (39.50 ppm in DMSO-$d_6$) for carbon spectra. Attributions were done by means of chemical shifts, peak integration, attached proton test (APT), COSY ($^1$H-$^{13}$C), hydrogen multiple bond coherence (HMBC) experiments, dynamic NMR spectra, selective deuteration, and model spectra. Elemental analyses were done by combustion, for C, H, N, with an automated Carlo Erba analyzer, and the results were found to be ±0.4% within the theoretical values.

All reactions were monitored by thin-layer chromatography (TLC), using 0.25 mm thick precoated silica gel plates (E. Merck) eluted with MeOH:CHCl$_3$ 1:4 v/v. 3-Aminopyrazole, 2-amino-imidazole sulfate, and 5-amino-tetrazole hydrate were from Sigma-Aldrich (Milan, Italy). Pyrylium salts were prepared by literature procedures, optimized in our laboratories.[15]

**General Procedure for the Preparation of Compounds 4–34.** Amino-azole (5 mmol) and the appropriate pyrylium salt (6 mmol) were suspended in 10 mL of anhydrous methanol. In the case of 2-amino-imidazole, the free amine was obtained as methanolic solution by refluxing the corresponding sulfate (Sigma) with the stoichiometric amount of MeONa in MeOH for 1 h, followed by filtration of the precipitated Na$_2$SO$_4$.

Triethylamine (6 mmol) was added, and the obtained solution was refluxed under stirring for 10 min, then AcOH (12 mmol) was added, and the mixture was refluxed for another 3–4 h. After that time, reflux was discontinued, 0.5 mL of concentrated NH$_3$ solution was added, and the solution was heated again for 5 min in order to convert the unreacted pyrylium salt into the corresponding pyridine. After cooling, the final reaction mixture was poured in 80 mL of diethyl ether, when crystallization usually occurred. The crude product was filtered, washed with ether, and recrystallized from alcohols (MeOH or EtOH/$^i$PrOH mixtures). Yields were in the range of 15–98%.

**General Procedure for the Preparation of Compounds 35–45.** 5-Aminotetrazole hydrate (5 mmol) and the appropriate pyrylium salt (6 mmol) were suspended in 3 mL of anhydrous methanol and poured into a stirred mixture of 25 mmol triethylamine and 15 mmol acetic anhydride previously cooled at 0 °C (ice bath). After 5 min of stirring, 10 mL of methanol were added, and the mixture was heated to reflux for 10 min. Then 15 mmol of acetic acid was added, and the mixture was refluxed for 4 h. The workup was the same as in case of compounds **4–34**. In several cases, the purified compounds still contained variable amounts of ammonium salts which co-crystallized with the desired material. For eliminating these salts, the crude products were suspended in 5 mL of distilled water and treated dropwise with 10% aqueous NaOH under stirring, until pH 13 was reached, filtered, washed with water and 5% aqueous HClO$_4$ solution, and recrystallized again. Yields were in the range of 10–84%.

The new derivatives **4–45** were characterized by elemental analysis and detailed spectroscopic data (IR and $^1$H/$^{13}$C NMR spectra). In some cases, advanced NMR techniques were used for complete structural attributions, i.e., selective decoupling, bidimensional correlations spectra COSY ($^1$H-$^{13}$C), and dynamic NMR spectroscopy. These experiments also permitted the evaluation of the barrier to rotation around the sp$^2$-sp$^2$ N$_{pyridinium}$-C$_{azole}$ bond, data that will be reported elsewhere.

**1-(Pyrazol-3-yl)-2,6-dimethyl-4-phenyl-pyridinium perchlorate, 8:** cream crystals, mp 246–8 °C (EtOH–MeOH 1:1 v/v). FTIR (KBr), cm$^{-1}$: 624 (ClO$_4^-$), 776, 974, 1082 (ClO$_4^-$), 1221, 1299, 1334, 1380, 1455, 1473, 1529, 1558, 1595, 1634, 2881, 2947, 2967, 3021, 3076, 3145, 3256, 3373; $^1$H NMR (DMSO-$d_6$), $\delta$, ppm, $J$, Hz: 13.83 (bs, 1H, NH, deuterable), 8.47 (s, 2H, H$_\beta$ of Py$^+$), 8.20 (d, $^3J_{HH}$ = 2.4, 1H, H-3 of pyrazole),

8.11 (dd, 2H, $H_{ortho}$ of Phe, $^3J_{HH}$ = 7.3, $^4J_{HH}$ = 2.3), 7.68 (m, 3H, $H_{meta}$ of Phe + $H_{para}$ of Phe), 6.71 (d, $^3J_{HH}$ = 2.4, 1H, *H*-4 of pyrazole), 2.48 (s, 6H, C$H_3$); $^{13}$C NMR (DMSO-$d_6$), $\delta$, ppm: 156.99 (*C*q, $C_\alpha$ of Py$^+$), 155.88 (*C*q, $C_\gamma$ of Py$^+$), 145.26 (*C*q, *C*-3 of pyrazole), 133.55 (*C*q, $C_{ipso}$ of Phe), 132.66 (*C*H, *C*-5 of pyrazole), 132.66 (*C*H, $C_{para}$ of Phe), 129.77 (*C*H, $C_{meta}$ of Phe), 128.30 (*C*H, $C_{ortho}$ of Phe), 123.22 (*C*H, $C_\beta$ of Py$^+$), 101.96 (*C*H, *C*-4 of pyrazole), 21.08 (*C*H$_3$). Anal. (C$_{16}$H$_{16}$N$_3$$^+$ClO$_4$$^-$) C, H, N.

**1-(Imidazol-2-yl)-2,6-dimethyl-4-phenyl-pyridinium perchlorate, 23:** cream crystals, mp 246–8 °C (EtOH–MeOH 1:1 v/v). FTIR (KBr), cm$^{-1}$: 625 (ClO$_4$$^-$), 772, 803, 884, 1094 (ClO$_4$$^-$), 1216, 1294, 1333, 1376, 1449, 1473, 1539, 1559, 1590, 1633, 2845, 2888, 2920, 3049, 3109, 3180; $^1$H NMR (DMSO-$d_6$), $\delta$, ppm, *J*, Hz: 8.53 (s, 2H, $H_\beta$ of Py$^+$), 8.16 (dd, 2H, $H_{ortho}$ of Phe, $^3J_{HH}$ = 7.1, $^4J_{HH}$ = 2.1), 7.71 (m, 3H, $H_{meta}$ of Phe + $H_{para}$ of Phe), 7.43 (s, 2H, C*H* of imidazole), 2.47 (s, 6H, C$H_3$); $^{13}$C NMR (DMSO-$d_6$), $\delta$, ppm: 157.49 (*C*q, $C_\alpha$ of Py$^+$), 157.10 (*C*q, $C_\gamma$ of Py$^+$), 135.70 (*C*q, *C*-2 of imidazole), 133.33 (*C*q, $C_{ipso}$ of Phe), 132.92 (*C*H, $C_{para}$ of Phe), 129.88 (*C*H, $C_{meta}$ of Phe), 128.54 (*C*H, $C_{ortho}$ of Phe), 124.37 (*C*H, *C*-4,5 of imidazole), 123.25 (*C*H, $C_\beta$ of Py$^+$), 20.19 (*C*H$_3$). Anal. (C$_{16}$H$_{16}$N$_3$$^+$ClO$_4$$^-$) C, H, N.

**1-(Tetrazol-5-ylate)-2,6-dimethyl-4-phenyl-pyridinium, 35:** cream crystals, mp 279–81 °C (desc.) (MeOH). FTIR (KBr), cm$^{-1}$: 606, 694, 770, 878, 997, 1009, 1022, 1063, 1081, 1128, 1146, 1190, 1336, 1380, 1404, 1457, 1559, 1635, 2846, 2928, 3083, 3227, 3351; $^1$H NMR (DMSO-$d_6$), $\delta$, ppm, *J*, Hz: 8.49 (s, 2H, $H_\beta$ of Py$^+$), 8.12 (dd, 2H, $H_{ortho}$ of Phe, $^3J_{HH}$ = 7.2, $^4J_{HH}$ = 2.2), 7.68 (m, 3H, $H_{meta}$ of Phe + $H_{para}$ of Phe), 2.36 (s, 6H, C$H_3$); $^{13}$C NMR (DMSO-$d_6$), $\delta$, ppm: 157.26 (*C*q, *C*-5 of tetrazole), 156.37 (*C*q, $C_\alpha$ of Py$^+$), 155.85 (*C*q, $C_\gamma$ of Py$^+$), 133.56 (*C*q, $C_{ipso}$ of Phe), 132.41 (*C*H, $C_{para}$ of Phe), 129.66 (*C*H, $C_{meta}$ of Phe), 128.31 (*C*H, $C_{ortho}$ of Phe), 123.27 (*C*H, $C_\beta$ of Py$^+$), 20.51 (*C*H$_3$). Anal. (C$_{14}$H$_{14}$N$_4$) C, H, N.

**Enzyme Preparations.** Human CA I and CA II cDNAs were expressed in *Escherichia coli* strain BL21 (DE3) from the plasmids pACA/hCA I and pACA/hCA II described by Lindskog et al.[27] (the two plasmids were a gift from Prof. Sven Lindskog, Umea University, Sweden). Cell growth conditions were those described by this group,[28] and enzymes were purified by affinity chromatography according to the method of Khalifah et al.[29] Enzyme concentrations were determined spectrophotometrically at 280 nm, utilizing a molar absorptivity of 49 mM$^{-1}$ cm$^{-1}$ for CA I and 54 mM$^{-1}$ cm$^{-1}$ for CA II, respectively, based on $M_r$ = 28.85 kDa for CA I and 29.30 kDa for CA II, respectively.[30] CA IV was isolated from bovine lung microsomes as described by Maren et al., and its concentration has been determined by titration with ethoxzolamide.[31]

Initial rates of 4-nitrophenyl acetate hydrolysis catalyzed by different CA isozymes were monitored spectrophotometrically, at 400 nm, with a Cary 3 instrument interfaced with an IBM-compatible PC.[25] Solutions of substrate were prepared in anhydrous acetonitrile; the substrate concentrations varied between $2 \times 10^{-2}$ and $1 \times 10^{-6}$ M, working at 25 °C. A molar absorption coefficient $\epsilon$ of 18 400 M$^{-1}$ cm$^{-1}$ was used for the 4-nitrophenolate formed by hydrolysis, in the conditions of the experiments (pH 7.40), as reported in the literature.[25] Nonenzymatic hydrolysis rates were always subtracted from the observed rates. Duplicate experiments were done for each activator concentration, and the values reported throughout the paper are the mean of such results. Stock solutions of activator (1 mM) were prepared in distilled–deionized water with 10–15% (v/v) DMSO (which is not inhibitory/activatory at these concentrations) and dilutions up to 0.1 nM were done thereafter with distilled–deionized water. Activator and enzyme solutions were preincubated together for 15 min at room temperature prior to assay, in order to allow for the formation of the E–A complex. The activation constant $K_A$ was determined as described by Briganti et al.[2] Enzyme concentrations were 7 nM for hCA II, 25 nM for hCA I, and 33 nM for bCA IV (this isozyme has a decreased esterase activity,[32] and higher concentrations had to be used for the measurements).

**Ex Vivo CA Activation.** An amount of 2 mL of freshly isolated human blood was thoroughly washed several times with 5 mL of Tris buffer (pH 7.40, 5 mM) and centrifuged for 10 min. The obtained erythrocytes were then treated with 2 mL of a 5 $\mu$M solution of CA activator. Incubation has been done at 37 °C with gentle stirring, for periods of 30–60 min. After that time, the red cells were centrifuged again for 10 min, the supernatant discarded, and the cells washed three times with 5 mL of the above-mentioned buffer, to eliminate all unbound compound. The cells were then lysed in 5 mL of distilled water and centrifuged for eliminating membranes and other insoluble materials, and the CA activity has been assayed as described above. Blank experiments were done in which no activator has been added to the blood red cells treated as described above, and CA activity determined in such conditions has been taken as 100%.[23,26] The same type of experiment was then repeated, lysing the red blood cells first, and incubating the obtained supernatant with the activators as described above.

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