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Laboratory note

Carbonic anhydrase activators. Part 17. Synthesis and activation study of a series of 1-(1,2,4-triazole-(1H)-3-yl)-2,4,6-trisubstituted-pyridinium salts against isozymes I, II and IV

MA Ilies[1], MD Banciu[2], M Ilies[1], F Chiraleu[3], F Briganti[4], A Scozzafava[4], CT Supuran[4]*

[1]*University of Bucharest, TH Maren Laboratory of Bio-organic and Bioinorganic Chemistry, Bd Republicii 13;*
[2]*Polytechnic University, Department of Organic Chemistry, Splaiul Independentei 313;*
[3]*Roumanian Academy, CD Nenitzescu Institute of Organic Chemistry, Splaiul Independentei 202, Bucharest, Roumania;*
[4]*Università degli Studi, Dipartimento di Chimica, Laboratorio di Chimica Inorganica e Bioinorganica, Via Gino Capponi 7, 50121 Firenze, Italy*

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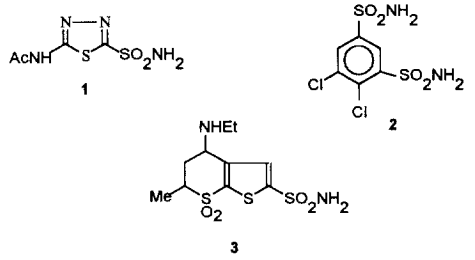
Summary — A series of pyridinium salts were prepared by reaction of 3-amino-(1H)-1,2,4-triazole with 2,4,6-trisubstituted pyrylium salts. The new compounds have been characterized by standard procedures and were assayed for their interaction with three carbonic anhydrase (CA) isozymes, the cytosolic CA I and CA II, as well as the membrane-bound CA IV. The pyridinium salts activate the three isozymes in a different manner, with CA IV being the most susceptible to activation, followed by CA I, whereas CA II is the least sensitive to this class of activators. Compounds possessing 2,6-dialkyl-4-phenyl substituents at the pyridinium ring were the most effective activators. This is the first study of comparative activation for several CA isozymes, proving that activators, similarly to inhibitors possess isozyme-specificity.

triazole / carbonic anhydrase, isozyme I, II, IV / pyrylium salt / pyridinium salt

Introduction

Inhibitors of the enzyme carbonic anhydrase (CA, EC 4.2.1.1) of the sulfonamide type, such as acetazolamide **1**, dichlorophenamide **2** or dorzolamide (Trusopt®) **3**, are widely used clinical agents [1, 2] in the management of diverse disorders. Their mechanism of action at molecular level is well understood: similarly to anionic inhibitors, such as cyanide, thiocyanate, azide, etc [3–5] sulfonamides bind as RSO_2NH^- anions to the metal ion within the active site of the diverse isozymes presently known (at least eight were isolated in higher vertebrates [2]), substituting the metal-bound water molecule/hydroxide ion [2, 6].



In contrast to inhibitors, activators of CAs were less studied, primarily due to the prejudice that a very rapid and efficient enzyme such as CA, should not need to be activated [7]. Still, CA II activation by phosphorylation in the presence of protein kinases and cAMP has been reported [8], these results having been reconfirmed more recently [9], showing that catecholamines activate red cell CA II, presumably by a mechanism involving phosphorylation of an active site threonine residue. Some anionic activators of CA III have also been reported [10, 11], the effect

*Correspondence and reprints
Abbreviations: CA – carbonic anhydrase; hCA – human CA; bCA – bovine CA

being explained as due to the proton shuttling capacities of such molecules. On the other hand, one of us [12–19] studied the activation of CA II and CA I by amines and amino acids, proposing a general scheme that should explain the activation mechanisms for isozymes CA I–III [12].

Normally, the catalytic process for the physiological reaction catalyzed by CA-s – CO_2 hydration – involves the nucleophilic attack of zinc-bound hydroxide to CO_2, fixed and optimally oriented in the hydrophobic pocket of the enzyme [20]. Bicarbonate formed in this way is then replaced by a water molecule, with generation of the catalytically inactive form of the enzyme EZn^{2+}–OH_2 (equation (1)). In order to regenerate the catalytically active one, a proton transfer reaction must occur, from the water bound to Zn(II) within the enzyme active site, to the external medium. In isozyme CA II, this step (equation (2)) was considered to be assisted by the active site residue His 64 [21], placed at the entrance of the active site, as well as by external buffer molecules [20, 21]. This step is also rate-determining for the whole catalytic cycle [22] and the shuttling effects of His 64 would explain the very high efficiency of CA II as catalyst, with a maximal turnover number of 1.6×10^6 s^{-1} [2, 20].

$$EZn^{2+} - HO^- + CO_2 \Longleftrightarrow$$

$$EZn^{2+} - HCO_3^- \overset{H_2O}{\Longleftrightarrow} EZn^{2+} - OH_2 + HCO_3^- \quad (1)$$

$$EZn^{2+} - OH_2 \Longleftrightarrow EZn^{2+} - HO^- + H^+ \quad (2)$$

In the presence of activators, an enzyme–activator complex is formed (similarly to the enzyme–inhibitor adducts, but without substitution of the metal bound solvent molecule), in which the activator bound within the active site facilitates proton transfer processes [12–17]. The driving force of this effect might be the fact that intramolecular reactions are more rapid than intermolecular ones [23]. Thus, in the presence of activators (symbolized as 'A'), equation (2) becomes (3):

$$EZn^{2+} - OH_2 + A \Longleftrightarrow [EZn^{2+} - OH_2 - A] \Longleftrightarrow$$
$$[EZn^{2+} - HO^- - AH^+] \Longleftrightarrow EZn^{2+} - HO^- + AH^+ \quad (3)$$

enzyme–activator complexes

Recently, the above mechanism has been confirmed, by the report of the first X-ray crystallographic structure of a CA–activator adduct by Briganti et al [24]. In figure 1 the binding of histamine to hCA II is shown. This figure has been generated from the crystallographic coordinates of the adduct, obtained by our group [24], and it clearly indicates that the activator molecule is anchored within the enzyme active site, between residues His 64 and Gln 92, by means of hydrogen bonds involving amino acid residues and water molecules from the active site. Particularly important for binding is residue Gln 92, which forms a strong hydrogen bond with a nitrogen atom of the imidazolic moiety of histamine.

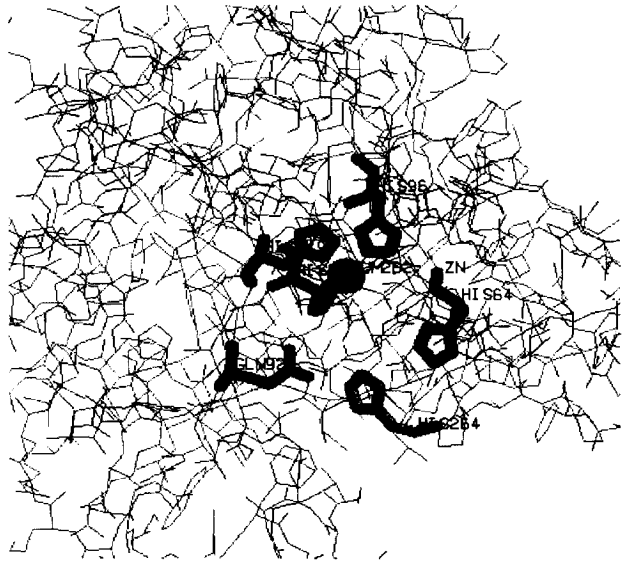
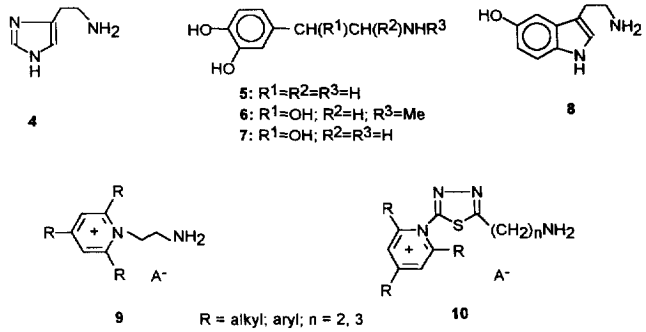


Fig 1. HCA II–histamine adduct: the Zn(II) ion (sphere) and its three histidine ligands (His 94, His 96 and His 119) are shown at the center of the active site, whereas histamine (numbered as His 264) is situated at the entrance in it, between residues His 64 (right) and Gln 92 (left). The figure was generated from the X-ray coordinates of the adduct [24], by using the program RasMol for Windows 2.6. The coordinates of this structure are deposited in the Brookhaven Protein Database.

In addition to histamine **4**, other strong CA II and CA I activators include such autacoids as dopamine **5**, adrenaline **6**, noradrenaline **7** and serotonin **8** [13–19]. Positively charged derivatives such as **9** and **10**, obtained from different amines and pyrylium salts, have also been proved to act as efficient CA II activators by this group [17, 25].



Since compounds **4–8** are known for their important role as neurotransmitters or agonists for important biological receptors, involved in critical physiological/physiopathological processes [26], it appeared of interest to continue study in the field of CA activators derived from both natural as well as synthetic lead molecules. In a previous work [17] we reported the synthesis and CA II activatory properties of a series of pyridinium salts of type **10**, which behaved as quite efficient activators. Taking into account these facts, we report here the synthesis and activation studies for other such positively charged derivatives, prepared via the reaction of amines with pyrylium salts. Here we report the preparation of a series of 1-(1,2,4-triazole-(1H)-3-yl)-2,4,6-trisubstituted- and 2,3,4,6-tetrasubstituted-pyridinium salts, as well as their activatory properties against three CA isozymes, ie, CA I, II and IV. As far as we now, this is the first comparative activation study on different CA isozymes, which led to evidencing important differences between their behaviour and discrimination towards diverse classes of modulators. Furthermore, this is the first study in which CA IV activation has been investigated.

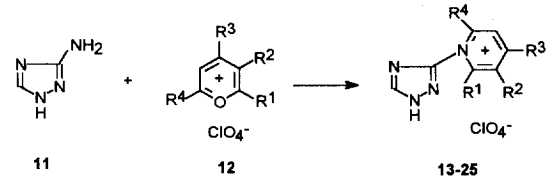
Results

Chemistry

Reaction of 3-amino-(1H)-1,2,4-triazole **11** with pyrylium salts **12** afforded the pyridinium salts **13–25** (scheme 1). The new compounds were characterized by standard analytic and spectroscopic methods that proved the proposed structure.

Pharmacology

The new activators were assayed against three CA isozymes (table I and figs 2–4) due to the fact that CA I, II and IV are the most abundant among the eight CA's isolated so far in higher vertebrates [2, 3, 26] on one hand, and on the other one, the high-activity forms CA II and CA IV are considered to play the key physiological role in many tissues in which CA activity is present [2, 3, 26]. Although hCA I is the second-most abundant protein in blood (after serum albumin), its physiological role is unknown [1, 2, 26]. However, this isozyme may be largely inhibited (around 85%) by the inorganic anions (such as chloride or phosphate) present in the plasma [2,3], but it is probably activated too, by the different autacoids present in the blood at high enough concentrations (such as histamine, catecholamines, serotonin, etc). Still, this activation process has never been considered from a physiological point of view up to now. For instance, it was recently proved by this group that a concentration as low as 10^{-7} M of histamine produces a 150% activation of hCA I [24]. Although CA II, one of the most powerful catalysts ever evolved in nature



Scheme 1.

Table I. Compounds **13–25** prepared in the present study and their CA activatory properties against isozymes CA I, II and IV, at 10^{-5} M activator concentration.

Compound	R^1	R^2	R^3	R^4	*% CA activity*[a]		
					hCA I[b]	*hCA II*[c]	*bCA IV*[d]
13	Me	H	Ph	Me	139	130	156
14	Et	H	Ph	Et	140	135	159
15	*n*-Pr	H	Ph	*n*-Pr	138	136	155
16	*i*-Pr	H	Ph	*i*-Pr	148	141	168
17	Ph	H	Ph	Ph	100	100	100
18	*i*-Pr	H	Me	*i*-Pr	139	125	157
19	*t*-Bu	H	Me	*t*-Bu	118	126	150
20	Me	Me	Ph	Me	142	128	160
21	Me	H	Ph	Ph	110	105	124
22	Et	H	Ph	Ph	105	100	121
23	*n*-Pr	H	Ph	Ph	100	100	115
24	*i*-Pr	H	Ph	Ph	100	100	105
25	*n*-Bu	H	Ph	Ph	100	100	100
11	–	–	–	–	83	67	75
4 (histamine)	–	–	–	–	169	134	155

[a]Control CA activity in the absence of activator is taken as 100%; [b][hCA I] = 0.03 μM; [c][hCA II] = 0.01 μM; [d][bCA IV] = 0.32 μM.

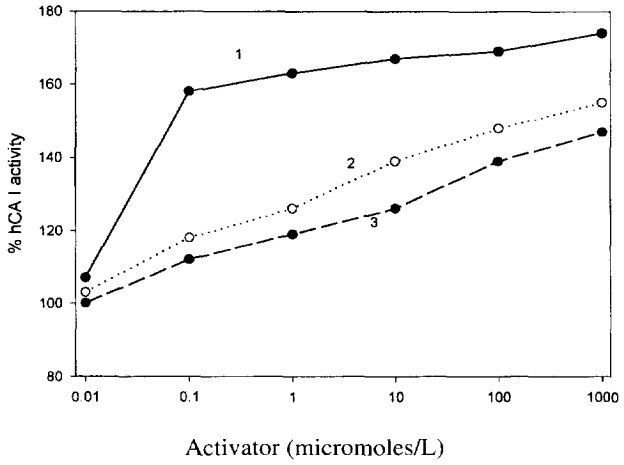


Fig 2. Activation of hCA I by histamine **4** (curve 1), compound **16** (curve 2) and compound **18** (curve 3), in the concentration range 10^{-8}–10^{-3} M. Conditions were: [hCA I] = 0.03 μM; substrate concentration 1 mM; 50 mM Tris buffer, pH 7.80, at 25 °C and ionic strength of 0.1(K_2SO_4). CA activity in the absence of activator is taken as 100%.

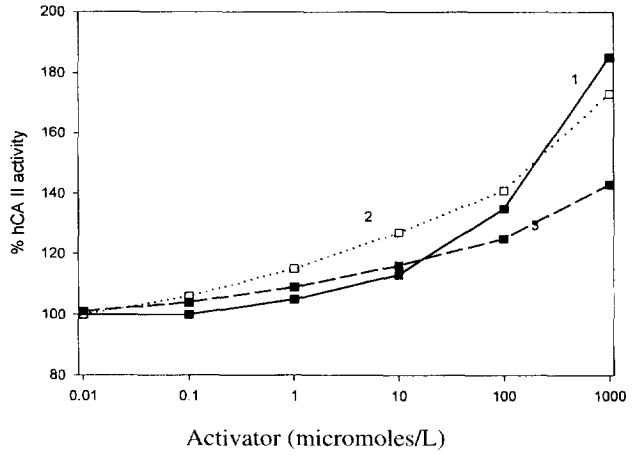


Fig 3. Activation of hCA II with histamine **4** (curve 1), compound **16** (curve 2) and compound **18** (curve 3), in the concentration range 10^{-8}–10^{-3} M. Conditions were: [hCA II] = 0.01 μM; substrate concentration 1 mM; 50 mM Tris buffer, pH 7.80, at 25 °C and ionic strength of 0.1 (K_2SO_4). CA activity in the absence of activator is taken as 100%.

[27] is not so abundant as CA I [1, 2], this high activity isozyme is virtually present in every cell [2, 3] where it catalyzes the hydration of CO_2 generated in metabolic processes. In many tissues (such as cilliary processes within the eye, gastric or pancreatic mucosa, kidneys, etc) it also participates in the processes of electrolyte secretion, generating H^+ or HCO_3^- ions (the products of CO_2 hydration) [2, 3]. Recently it was shown [28] that many processes in which CA II was thought to play the critical role, are in fact largely modulated by the CA IV activity. This isozyme is membrane-bound, being very abundant in the lung, kidney, gastro-intestinal tract and hepatocytes of vertebrates, among others [3, 28–31]. Like CA II, it is a high-activity form [28–31]. It is thus of considerable interest to search for activators (and obviously, also inhibitors) that would react predominantly with one or the other of these physiologically important enzymes. This should constitute an excellent means for assessing the physiological function of many of the presently known CA isozymes. In addition to the above-mentioned facts, the present one is the first study in which CA IV activation has been investigated.

Discussion

Reaction of tri-/tetra-substituted pyrylium salts **12** with 3-amino-(1H)-1,2,4-triazole **11** afforded the pyridinium salts **13–25**, with yields in the range of 18–75% (scheme 1 and table I). Pyrylium salts containing alkyl or aryl groups as well as a combination of the two types of groups were used in the synthesis, as it was previously established that the nature of these moieties strongly influences the biological activity as CA inhibitors/activators of the obtained derivatives [17, 32–35]. Surprisingly, the pyridinium salts containing 2,4,6-trimethyl and 2,3,4,6-tetramethyl moieties could not be purified satisfactorily in this case, and although the two compounds have been synthesized, they are not included in the list of derivatives **13–25**. Probably, cyclisation assisted by the 2-methyl group(s) of these derivatives led to the formation of the substituted anilino-triazole, in addition to the substituted pyridinium salt. The latter compound then could not be separated in pure form by simple recrystallisation from the reaction mixture.

The pyridinium salts containing 2,4,6-trimethyl and 2,4,6-triphenyl moieties were reported in a previous paper [36] but no experimental part or spectroscopic caracterisation was given.

Elemental analysis and spectroscopic data (IR-, electronic and ^{1}H-NMR) for the new compounds **13–25** confirmed the proposed structures (see *Experimental protocols* for details).

CA activation data with the new compounds **13–25**, histamine **4** as standard and 3-amino-(1H)-1,2,4-triazole **11** (the starting material in the synthesis) against three isozymes, hCA I, hCA II and bCA IV are shown in table I and figures 2–4.

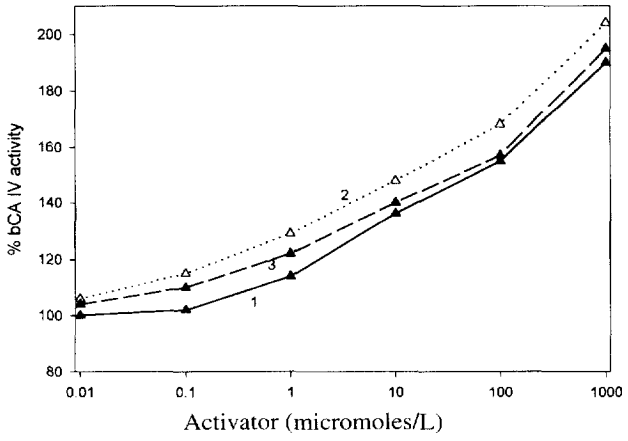


Fig 4. Activation of bCA IV with histamine **4** (curve 1), compound **16** (curve 2) and compound **18** (curve 3), in the concentration range 10^{-8}–10^{-3} M. Conditions were: [bCA IV] = 0.32 μM; substrate concentration 1 mM; 50 mM Tris buffer, pH 7.80, at 25 °C and ionic strength of 0.1 (K_2SO_4). CA activity in the absence of activator is taken as 100%.

Comparative studies regarding activation of diverse CA isozymes with different classes of activators have not been published up to now. Thus, surprising findings emerged during the present one. Data of table I and figures 2–4 show significant differences between the investigated isozymes in their behavior towards both 'classical' activators, such as histamine **4**, as well as the new class of activators synthesized in the present work. The most interesting finding is represented by the higher susceptibility of the membrane-bound isozyme CA IV to be activated by some of the pyridinium salts of type **13–25**, and the lower affinity of the rapid isozyme CA II for these compounds (compare figs 3 and 4). CA I on the other hand has an intermediate behavior towards them (fig 2), but it is much more susceptible to be activated by histamine, in contrast to the other two isozymes (for CA II an appreciable activation with histamine starts only at 1000 times higher activator concentrations whereas CA IV has an intermediate behavior). One should note that the CA activation is generally observed at higher compound concentrations as compared to the inhibition; thus, typically, strong CA inhibitors are effective in nanomolar concentrations, whereas a comparable activatory effect is seen only in micromolar concentrations. It is also to be noted that the amino-triazole **11**, used as starting material in the synthesis for the preparation of derivatives **13–25**, is not at all a CA activator, acting instead as a weak CA inhibitor (table I), similarly to triazole itself, which has recently been reported to inhibit CA II, and the X-ray crystal structure of its adduct with hCA II has also been published [34].

In addition to isozyme-specificity discussed above for this type of activators, it should be noted that the substitution pattern at the pyridinium ring of compounds **13–25** strongly influences their biological activity. Thus, compounds possessing 2,6-dialkyl-4-phenyl groups, such as **13–16**, were the most active against all three isozymes. Slightly less active were the 2,4,6-trialkyl-substituted derivatives **18**, **19**, whereas the 2,4-diaryl- and 2,4,6-triaryl-substituted compounds (**17**, **21–25**) were weakly active or totally devoid of activity. This might obviously be due to the very bulky nature of these last derivatives and as a consequence, their impaired access within the ezyme active site.

For the most active derivatives **13–16**, the nature of the alkyl 2,6-moieties of the pyridinium ring controls the activity as follows: increasing the chain from methyl to ethyl is beneficial for activity, but a further increase to *n*-propyl slightly reduces CA activatory properties. Still, the compound possessing two iso-propyl moieties, **16**, is the most active in the whole series of the new derivatives, and against all three CA isozymes.

The mechanism of action of these new CA activators probably involves facilitation of proton transfer reaction from the Zn(II)-bound water molecule to the reaction medium, via intermediates of the enzyme–activator type complexes, as in the case of previously investigated activators, of which histamine is the best studied [13–19]. It is thus interesting to note that starting from a weak CA inhibitor, 3-amino-(1H)-1,2,4-triazole **11**, it is possible to design quite effective CA activators for several isozymes. Probably the presence of the substituted pyridinium moiety in compounds **13–21** (the active derivatives in this series) has as a consequence that the imidazole type NH proton acquires a pK_a in the region 6–8, which was previously shown to be the pK_a interval beneficial for strong CA activatory properties, by this and Elguero's groups [12, 15, 16, 18]. The nature of the groups substituting the pyridinium moiety is thus important not only for assuring a relatively strong binding of the activator to the enzyme, but also in controlling the pK_a of the proton-transfer group of the activator molecule.

In conclusion, a novel class of CA activators, the 1-(1,2,4-triazole-(1H)-3-yl)-2,4,6-trisubstituted pyridinium salts is reported, together with the first comparative activation study on three isozymes, CA I, II and IV. Important differences in activation of these isozymes have been evidenced for the new type of activators, and the classical activator histamine.

Experimental protocols

Chemistry

The melting points were determined on a Boetius PHMK heating-plate microscope and are not corrected. UV spectra were obtained in methanolic solution, with a SPECORD UV-VIS Carl Zeiss Jena spectrophotometer; IR spectra in KBr pellets, with a double-beam UR-20 Carl Zeiss Jena spectrophotometer; ^{1}H-NMR spectra were recorded with a Varian A60A spectrometer or a Bruker CPX-100 spectrometer, using trifluoroacetic acid (TFA) as solvent. Chemical shifts are reported as parts per million, relative to Me_4Si as internal standard. Elemental analyses (C, H, N) were performed by microcombustion, with an automatic Carlo Erba analyser and the values obtained were within ±0.4% of the theoretical values.

3-Amino-(1H)-1,2,4-triazole **11** and solvents were from Merck and were used without further purification. Pyrylium salts **12** were prepared by literature procedures [32, 35].

Human CA I (hCA I) and CA II (hCA II) cDNAs were expressed in *Escherichia coli* strain BL21 (DE3) from the plasmids pACA/hCA I and pACA/hCA II as described in the literature [37] (the two plasmids were a gift from Prof Sven Lindskog, Umeå University, Sweden). Cell growth conditions were those described by Lindskog's group [38] and enzymes were purified by affinity chromatography according to the published method [39]. Enzyme concentrations were determined spectrophotometrically at 280 nm, utilizing a molar absorptivity of 49 mM^{-1} cm^{-1} for hCA I and 54 mM^{-1} cm^{-1} for hCA II, respectively, based on M_r = 28.85 kDa for CA I, and 29.30 kDa for CA II, respectively [40, 41]. bCA IV was isolated from bovine lung microsomes as described by Maren et al [28].

*General procedure for the preparation of compounds **13–25***

3-Amino-(1H)-1,2,4-triazole **11** (6 mmol) and the appropriate pyrylium salt **12** (6 mmol) were suspended in 20 mL of absolute methanol. Triethylamine (6 mmol) was added and the obtained solution was stirred for 5 min at room temperature, then AcOH (12 mmol) was added and the mixture was refluxed for 3–4 h. A part of the solvent was then evaporated under reduced pressure and the concentrated solution was poured into 150 mL of diethylether. The tarry product that usually formed was washed with dietylether, then extracted several times with 10 mL of 10% aqueous ammonia solution. The neutralisation of these solutions with 10% $HClO_4$ gave the crude products **13–25**.

Advanced purification has been achieved by dissolving the crude product in the minimum amount of 10% aqueous ammonia. The hot solution obtained in this way was then treated with activated charcoal and after filtration the desired compound was precipitated with 10% aqueous $HClO_4$. After filtration and drying over KOH the new compounds were recrystallized from methanol. Yields were in the range of 18–75%.

*1-(1,2,4-Triazole-(1H)-3-yl)-2,6-dimethyl-4-phenylpyridinium perchlorate **13***
Mp 314–316 °C. UV (MeOH): 215 nm, lg ε = 4.32; 231 nm, lg ε = 3.90; 299 nm, lg ε = 4.57. IR (KBr), cm^{-1}: (underlined bands are due to the anion) *625*, 650, 685, 765, 990, *1100*, 1220, 1250, 1330, 1390, 1450, 1470, 1520, 1560, 1595, 1630, 2920, 2980, 3090, 3140. ^{1}H-NMR (TFA), ppm: 2.73 (s, 6H, 2,6-diMe); 7.58–8.13 (m, 6H, Ph + H-N-1-triazole), 8.21 (s, 2H, 3,5-H-pyridinium); 9.28 (s, 1H, H-C-triazole). Anal $C_{15}H_{15}N_4^+ \bullet ClO_4^-$ (C, H, N).

*1-(1,2,4-Triazole-(1H)-3-yl)-2,6-diethyl-4-phenylpyridinium perchlorate **14***
Mp 275–277 °C. UV (MeOH): 235 nm, lg ε = 3.77; 297 nm, lg ε = 4.41. IR (KBr), cm^{-1}: *625*, 680, 765, 990, *1100*, 1250, 1310, 1335, 1380, 1430, 1455, 1520, 1560, 1595, 1630, 2880, 2940, 2990, 3080, 3110, 3140. ^{1}H-NMR (TFA), ppm: 1.49 (tr, 6H, 2 CH_3CH_2-); 2.88 (qu, 4H, 2 CH_3CH_2-); 7.59–8.23 (m, 6H, Ph + H-N-1-triazole); 8.28 (s, 2H, 3,5-H-pyridinium); 9.38 (s, 1H, H-C-triazole). Anal $C_{17}H_{19}N_4^+ \bullet ClO_4^-$ (C, H, N).

*1-(1,2,4-Triazole-(1H)-3-yl)-2,6-di-n-propyl-4-phenylpyridinium perchlorate **15***
Mp 175–177 °C. UV (MeOH): 237 nm, lg ε = 3.92; 296 nm, lg ε = 4.33. IR (KBr), cm^{-1}: *625*, 690, 770, *1100*, 1245, 1360, 1430, 1465, 1530, 1550, 1590, 1630, 2880, 2940, 2980, 3080. ^{1}H-NMR (TFA), ppm: 1.00 (tr, 6H, 2 $CH_3CH_2CH_2$-); 1.82 (hx, 4H, 2 $CH_3CH_2CH_2$-); 2.70 (tr, 4H, 2 $CH_3CH_2CH_2$-); 7.16–7.96 (m, 6H, Ph + H-N-1-triazole); 8.05 (s, 2H, 3,5-H-pyridinium); 9.08 (s, 1H, H-C-triazole). Anal $C_{19}H_{23}N_4^+ \bullet ClO_4^-$ (C, H, N).

*1-(1,2,4-Triazole-(1H)-3-yl)-2,6-di-iso-propyl-4-phenylpyridinium perchlorate **16***
Mp 209–210 °C. UV (MeOH): 243 nm, lg ε = 4.38; 300 nm, lg ε = 3.76. IR (KBr), cm^{-1}: *625*, 695, 780, *1100*, 1210, 1350, 1440, 1470, 1535, 1595, 1640, 2880, 2930, 2985, 3080. ^{1}H-NMR (TFA), ppm: 1.61 (d, 12H, 4CH_3 from 2,6-di-i-Pr); 3.61 (hp, 2H, 2CH from 2,6-di-i-Pr); 7.50–8.15 (m, 6H, Ph + H-N-1-triazole); 8.21 (s, 2H, 3,5-H-pyridinium); 9.74 (s, 1H, H-C-triazole). Anal $C_{19}H_{23}N_4^+ \bullet ClO_4^-$ (C, H, N).

*1-(1,2,4-Triazole-(1H)-3-yl)-2,4,6-triphenylpyridinium perchlorate **17***
Mp 269–271 °C. UV (MeOH): 240 nm, lg ε = 4.11; 322 nm, lg ε = 4.46. IR (KBr), cm^{-1}: *625*, 695, 760, *1100*, 1250, 1350, 1410, 1430, 1450, 1470, 1490, 1550, 1595, 1620, 3060, 3145, 3290. ^{1}H-NMR (TFA), ppm: 7.41–8.43 (m, 16H, 3Ph + H-N-1-triazole); 8.56 (s, 2H, 3,5-H-pyridinium); 9.03 (s, 1H, H-C-triazole). Anal $C_{25}H_{19}N_4^+ \bullet ClO_4^-$ (C, H, N).

*1-(1,2,4-Triazole-(1H)-3-yl)-2,6-di-iso-propyl-4-methylpyridinium perchlorate **18***
Mp 173–175 °C. UV (MeOH): 212 nm, lg ε = 3.50; 238 nm, lg ε = 3.54; 299 nm, lg ε = 3.97. IR (KBr), cm^{-1}: *625*, 920, 950, *1100*, 1200, 1225, 1245, 1330, 1375, 1410, 1470, 1500, 1535, 1630, 1680, 2880, 2940, 2980, 3060. ^{1}H-NMR (TFA), ppm: 1.40 (s, 12H, 4CH_3 from 2,6-di-i-Pr); 2.65 (s, 3H, 4-Me); 3.30 (hp, 2H, 2CH from 2,6-di-i-Pr); 7.10 (s, 1H, H-N-1-triazole); 7.62 (s, 2H, 3,5-H-pyridinium); 9.10 (s, 1H, H-C-triazole). Anal $C_{14}H_{21}N_4^+ \bullet ClO_4^-$ (C, H, N).

*1-(1,2,4-Triazole-(1H)-3-yl)-2,6-di-t-butyl-4-methylpyridinium perchlorate **19***
Mp 244–246 °C. UV (MeOH): 237 nm, lg ε = 3.88; 278 nm, lg ε = 4.06. IR (KBr), cm^{-1}: *625*, 925, *1100*, 1200, 1225, 1250, 1370, 1410, 1450, 1470, 1535, 1625, 2880, 2915, 2980, 3060. ^{1}H-NMR (TFA), ppm: 0.96 (s, 18H, 2,6-di-t-Bu); 2.64 (s, 3H, 4-Me); 7.14 (s, 1H, H-N-1-triazole); 7.60 (s, 2H, 3,5-H-pyridinium); 9.08 (s, 1H, H-C-triazole). Anal $C_{16}H_{25}N_4^+ \bullet ClO_4^-$ (C, H, N).

*1-(1,2,4-Triazole-(1H)-3-yl)-2,3,6-trimethyl-4-phenylpyridinium perchlorate **20***
Mp 266–269 °C. UV (MeOH): 237 nm, lg ε = 3.87; 292 nm, lg ε = 4.35. IR (KBr), cm^{-1}: *625*, 640, 705, 740, 760, 975, *1100*, 1260, 1325, 1340, 1390, 1450, 1510, 1540, 1620, 2980, 3100, 3140, 3280. ^{1}H-NMR (TFA), ppm: 2.61 (s, 3H, 3-Me); 2.65 (s,

6H, 2,6-di-Me); 7.49–7.91 (m, 6H, Ph + H-N-1-triazole); 7.95 (s, 1H, 5-H-pyridinium); 9.35 (s, 1H, H-C-triazole). Anal $C_{16}H_{17}N_4^+ \bullet ClO_4^-$ (C, H, N).

1-(1,2,4-Triazole-(1H)-3-yl)-2-methyl-4,6-diphenylpyridinium perchlorate ***21***
Mp 266–268 °C. UV (MeOH): 209 nm, lg ε = 4.51; 233 nm, lg ε = 4.40; 311 nm, lg ε = 4.48. IR (KBr), cm^{-1}: *625*, 680, 700, 770, *1100*, 1235, 1275, 1340, 1415, 1450, 1470, 1550, 1590, 1620, 2910, 2980, 3050, 3110. ^{1}H-NMR (TFA), ppm: 2.85 (s, 3H, 2-Me); 7.46–8.35 (m, 11H, 4,6-di-Ph + H-N-1-triazole); 8.43 (s, 1H, 3-H-pyridinium); 8.50 (s, 1H, 5-H-pyridinium); 9.12 (s, 1H, H-C-triazole). Anal $C_{20}H_{17}N_4^+ \bullet ClO_4^-$ (C, H, N).

1-(1,2,4-Triazole-(1H)-3-yl)-2-ethyl-4,6-diphenylpyridinium perchlorate ***22***
Mp 268–271 °C. UV (MeOH): 209 nm, lg ε = 4.39; 225 nm, lg ε = 4.16; 313 nm, lg ε = 4.50. IR (KBr), cm^{-1}: *625*, 675, 700, 770, *1100*, 1245, 1275, 1340, 1410, 1430, 1450, 1470, 1550, 1590, 1620, 2880, 2940, 2980, 3075, 3130. ^{1}H-NMR (TFA), ppm: 1.56 (tr, 3H, *CH*$_3$CH$_2$-); 3.11 (qu, 2H, CH$_3$*CH*$_2$-); 7.52–8.40 (m, 11H, 4,6-di-Ph + H-N-1-triazole); 8.51 (s, 1H, 3-H-pyridinium); 8.56 (s, 1H, 5-H-pyridinium); 9.18 (s, 1H, H-C-triazole). Anal $C_{21}H_{19}N_4^+ \bullet ClO_4^-$ (C, H, N).

1-(1,2,4-Triazole-(1H)-3-yl)-2-n-propyl-4,6-diphenylpyridinium perchlorate ***23***
Mp 260–263 °C. UV (MeOH): 207 nm, lg ε = 4.25; 227 nm, lg ε = 4.05; 311 nm, lg ε = 4.41. IR (KBr), cm^{-1}: *625*, 695, 770, *1100*, 1255, 1340, 1410, 1430, 1450, 1465, 1515, 1550, 1595, 1620, 2880, 2935, 2970, 3070, 3145, 3370. ^{1}H-NMR (TFA), ppm: 1.12 (tr, 3H, *CH*$_3$CH$_2$CH$_2$-); 2.02 (hx, 2H, CH$_3$*CH*$_2$CH$_2$-); 3.05 (tr, 2H, CH$_3$CH$_2$*CH*$_2$-); 7.38–8.40 (m, 11H, 4,6-di-Ph + H-N-1-triazole); 8.43 (s, 1H, 3-H-pyridinium); 8.52 (s, 1H, 5-H-pyridinium); 9.25 (s, 1H, H-C-triazole). Anal $C_{22}H_{12}N_4^+ \bullet ClO_4^-$ (C, H, N).

1-(1,2,4-Triazole-(1H)-3-yl)-2-iso-propyl-4,6-diphenylpyridinium perchlorate ***24***
Mp 241–244 °C. UV (MeOH): 208 nm, lg ε = 4.31; 227 nm, lg ε = 4.04; 311 nm, lg ε = 4.41. IR (KBr), cm^{-1}: *625*, 690, 775, *1100*, 1245, 1340, 1425, 1445, 1460, 1510, 1550, 1590, 1620, 2880, 2940, 2980, 3070, 3110, 3140, 3400. ^{1}H-NMR (TFA), ppm: 1.58 (d, 6H, 2CH$_3$ from i-Pr), 2.95 (hp, 1H, CH from i-Pr); 7.16–8.31 (m, 11H, 4,6-di-Ph + H-N-1-triazole); 8.43 (s, 1H, 3-H-pyridinium); 8.56 (s, 1H, 5-H-pyridinium); 9.28 (s, 1H, H-C-triazole). Anal $C_{22}H_{21}N_4^+ \bullet ClO_4^-$ (C, H, N).

1-(1,2,4-Triazole-(1H)-3-yl)-2-n-butyl-4,6-diphenylpyridinium perchlorate ***25***
Mp 239–241 °C. UV (MeOH): 208 nm, lg ε = 4.29; 227 nm, lg ε = 4.00; 311 nm, lg ε = 4.42. IR (KBr), cm^{-1}: *625*, 700, 770, *1100*, 1255, 1340, 1410, 1430, 1445, 1465, 1510, 1550, 1590, 1620, 2880, 2930, 2965, 3065, 3150, 3380. ^{1}H-NMR (TFA), ppm: 0.97 (tr, 3H, *CH*$_3$CH$_2$CH$_2$CH$_2$-); 1.19–2.39 (m, 4H, CH$_3$*CH*$_2$*CH*$_2$CH$_2$-); 2.98 (tr, 2H, CH$_3$CH$_2$CH$_2$*CH*$_2$-); 6.85–8.62 (m, 13H, 4,6-di-Ph + H-N-1-triazole + 3,5-H-pyridinium); 9.12 (s, 1H, H-C-triazole). Anal $C_{23}H_{23}N_4^+ \bullet ClO_4^-$ (C, H, N).

Pharmacology

Initial rates of 4-nitrophenyl acetate hydrolysis were monitored spectrophotometrically, at 400 nm, with a Cary 3 instrument interfaced with an IBM compatible PC [42]. Substrate solutions were prepared in anhydrous acetonitrile; the substrate concentrations varied between 2×10^{-2} and 1×10^{-6} M, working at 25 °C. A molar absorption coefficient ε of 18 400 M^{-1} cm^{-1} was used for the 4-nitrophenolate formed by hydrolysis, under the conditions of the experiments (pH 7.80), as reported in the literature [42]. Nonenzymic hydrolysis rates were always subtracted from the observed rates. Duplicate experiments were done for each activator, and the values reported throughout the paper are the mean of such results. The enzymatic rates (without activator) were obtained as the mean from at least three experiments.

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