Organic & Biomolecular Chemistry

Cite this: *Org. Biomol. Chem.*, 2011, **9**, 2790

www.rsc.org/obc

PAPER

Pyridinium derivatives of histamine are potent activators of cytosolic carbonic anhydrase isoforms I, II and VII†

Khyati Dave,[a,b] Andrea Scozzafava,[c] Daniela Vullo,[c] Claudiu T. Supuran*[c] and Marc A. Ilies*[a,b]

Received 10th September 2010, Accepted 5th January 2011
DOI: 10.1039/c0ob00703j

A series of positively-charged derivatives has been prepared by reaction of histamine with substituted pyrylium salts. These pyridinium histamine derivatives were investigated as activators of the zinc enzyme carbonic anhydrase (CA, EC 4.2.1.1) and more precisely the human isoforms hCA I, II and VII. Activities from the subnanomolar to the micromolar range were detected for these compounds as activators of the three isoforms, confirming the validity of current and previous designs. The substitution pattern at the pyridinium ring was the main factor influencing activity, the three isoforms showing different structural requirements for good activity, related with the number of pyridinium substituting groups and their nature, among various alkyl, phenyl and *para*-substituted styryl moieties. We were successful in identifying nanomolar potent and selective activators for each isozyme and also activators with a relatively good activity against all isozymes tested—valuable lead compounds for physiology and pathology studies involving these isozymes.

Introduction

Carbonic anhydrase (CA, EC 4.2.1.1) is a ubiquitous metalloenzyme present in prokaryotes and eukaryotes that speeds up the equilibration of CO_2 with HCO_3^- in water at neutral pH ($CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$). In higher vertebrates CA is extensively involved in respiration and CO_2 transport between the metabolizing tissues and lungs, pH and carbon dioxide homeostasis, electrolyte secretion in various tissues and organs, biosynthetic reactions, bone resorption, calcification, tumorigenicity, etc. through its 16 known isozymes.[1–5]

The active site of most CA isozymes contains a zinc ion (Zn^{2+}), which is essential for catalysis,[6] and it is used to decrease the pKa of a coordinated water molecule, facilitating its ionization in the rate-limiting step of the catalytic mechanism (equation 1).[7] The resulting proton is transferred from the active site to the external buffers *via* a proton shuttle (His64 in isoform CA II).[8] The zinc-bound hydroxide thus generated attacks the CO_2 molecule bound in a hydrophobic pocket nearby, generating bicarbonate. Subsequent displacement of HCO_3^- from the Zn^{2+} ion by another water molecule completes the catalytic cycle (equation 2).[7,9] Inorganic anions, primary aromatic and heterocyclic sulfonamides, sulfamates, sulfamides can bind to the zinc ion and inhibit the activity of the enzyme.[2,3,10–12] CA inhibition was studied intensely immediately after the enzyme discovery and sulfonamide CA inhibitors (CAIs) were exploited for more than five decades in the treatment of edema, hypertension, glaucoma, obesity, cancer, epilepsy and osteoporosis.[1–3,11]

$$EZn^{2+}\text{-}OH_2 \rightleftharpoons EZn^{2+}\text{-}OH^- + H^+ \quad \text{-rate determining step-} \qquad (1)$$

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

$$EZn^{2+}\text{-}OH_2 + A \rightleftharpoons [EZn^{2+}\text{-}OH_2\text{-}A] \rightleftharpoons [EZn^{2+}\text{-}OH^-\text{-}AH^+] \rightleftharpoons EZn^{2+}\text{-}OH^- + AH^+ \qquad (3)$$

enzyme-activator complexes

In contrast with CAIs, CA activators (CAAs) remained largely unexplored for a long time,[13,14] although activation of CA was reported simultaneously with its inhibition.[15] Contributions made by Ho and Sturtevant (activation of CA with EDTA[16]), Puscas *et al.* (activation of CA with histamine[17]), Silverman *et al.* (activation of CA isozymes with imidazole, phosphate, histidine, and hemoglobin[18–20]), and Chegwidden and Shelton (activation of CA III with phosphate[21]) built confidence towards the possibility of CA activation. In 1989, Parkes and Coleman[22] reinvestigated the lysine, polylysine, imidazole, glycine and histidine CA activation effects. CAAs intervene in the catalytic cycle (eqn (3)).

[a]*Temple University School of Pharmacy, Department of Pharmaceutical Sciences, 3307 North Broad Street, Philadelphia, PA-19140, USA . E-mail: mailies@temple.edu; Fax: +1-215-707-5620; Tel: +1-215-707-1749*

[b]*Moulder Center for Drug Discovery Research, Temple University, 3307 North Broad Street, Philadelphia, PA-19140, USA*

[c]*Università degli Studi, Department of Chemistry Ugo Schiff, Laboratory of Bioinorganic Chemistry, Via della Lastruccia 3, Rm. 188, Polo Scientifico, 50019-Sesto Fiorentino (Florence), Italy. E-mail: claudiu.supuran@unifi.it; Fax: +39-055-4573385; Tel: +39-055-4573005*

† Dedicated to Professor Alexandru T. Balaban, on the occasion of his 80th birthday anniversary

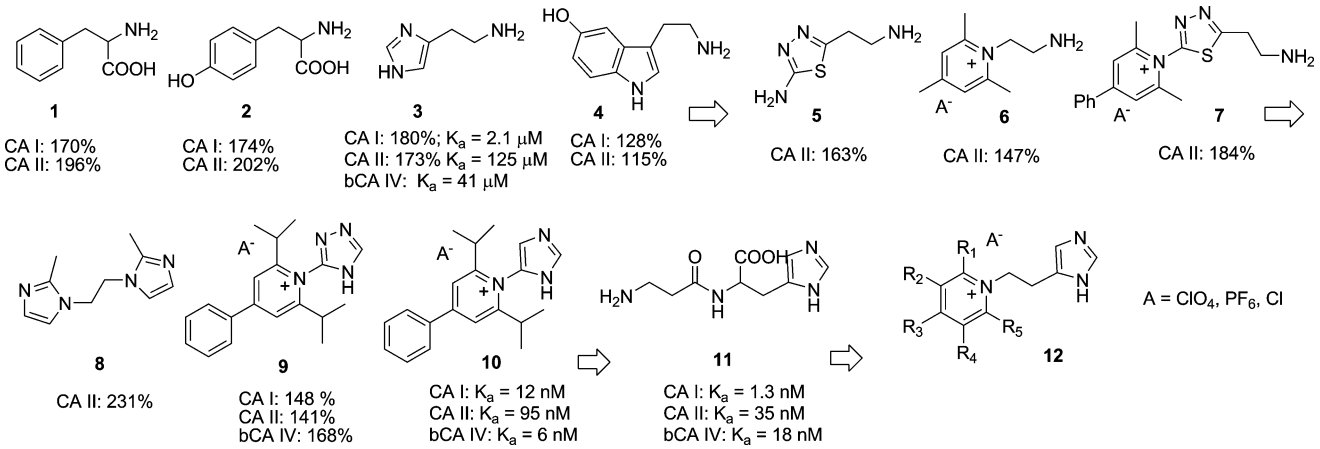


Chart 1 Evolution of CAAs' design, the activation potency of representative lead compounds **1–11** at a concentration of 10 μM against human CA I, CA II, and bovine CA IV,[13,24,26–28,30–33] and the proposed design for the novel CAAs (**12**).

These reports spurred extensive efforts towards discovery and validation of new CAAs. Many natural compounds incorporating basic moieties, such as biogenic amines, amino acids, and peptides were investigated and found to be CAAs of various potencies.[13,14,23,24] Thus, phenylalanine (**1**), tyrosine (**2**), and histamine (**3**) (Chart 1) were found to be very efficient CAAs for both isozyme CA I and CA II, all having a primary amine as proton shuttling group. Serotonin (**4**) was found to be moderately active. The amino group was retained in the design of second generation activators (**5–7**), incorporating heterocyclic amphiphilic anchors, among which the pyridinium moiety was particularly efficient.[25,26] Activation of CA with bis-azoles such as bis-imidazole (**8**) was discovered almost simultaneously. In these compounds one imidazole ring acts as hydrophobic anchor and the other one as proton shuttle (Chart 1).[27,28] These findings prompted the reinvestigation of CA activation with histamine (**3**), which possesses two moieties that can shuttle protons. X-ray crystallography of CA II in complex with histamine[29] revealed that the activator binds at the entrance of the active site cavity through the imidazole moiety which participates in shuttling protons between the active site and the bulk solvent, thus acting as a second proton shuttle of the enzyme in addition to His64, whereas the amino moiety from the aminoethyl group of histamine, does not participate in any interaction with the enzyme active site and might be thus derivatized.[29]

A comprehensive structure–activity relationship study[24] revealed that efficient CAAs possess a proton-shuttling group attached to a hydrophobic/amphiphilic aromatic/heterocyclic anchor *via* a short, flexible linker. Therefore various azoles have been subsequently tested as proton-shuttling moieties in combination with the most efficient anchors, yielding powerful activators, such as 1,2,4-triazole pyridinium derivative **9**.[30] However, a focused SAR study on various azoles in combination with the efficient cationic pyridinium anchor[31] revealed the imidazole moiety as the best proton shuttle (*e.g.*, compound **10**), due to a pKa in the maximum activity range for CA I, II, and IV (6.5–8.5 pKa units).[13] CA activation studies with various imidazole derivatives (histamine derivatives, 4-methylimidazole, D- and L-histidine, carnosine (**11**) and congeners, etc) subsequently confirmed this conclusion.[32–43]

One limitation of the pyridinium imidazole CAAs **10** and congeners is the reduced mobility of the azole ring, which is directly attached to the pyridinium moiety. The direct connection between the two heterocyclic systems has a significant influence on the electronic properties of both aromatic systems and on their relative conformations.[44] Since the positively-charged pyridinium group has a strong electron-withdrawing field effect, it is expected to diminish the pKa of the imidazole moiety and to reduce its proton shuttling capabilities. Building on these premises we are now reporting the synthesis and CA activation properties of a new series of pyridinium imidazoles **12** in which the pyridinium anchor is separated from the imidazole ring *via* a short two carbon linker. The design relies on the interesting activation properties of histamine **3** and the fact that the 3D structure of the adduct of CA II with this lead molecule is available.[29] The aim of this study was to synthesize and evaluate the effect of replacing the small amino group anchoring moiety with a bulkier pyridinium one on the activation of the cytosolic isozymes CA I, CA II and CA VII (all involved in crucial physiologic processes),[2] in a detailed structure–activity relationship (SAR) study.

Results and Discussion

Chemistry

The structural diversity of pyridinium substituents can be easily generated using a library of highly reactive pyrylium salts (Chart 2), conveniently prepared by acylation of mesityl oxide or diacylation of olefins (the Balaban–Nenitzescu–Praill reaction) and related procedures.[45] Pyrylium salts were reacted with histamine following the Bayer–Piccard reaction[46] and generated the corresponding pyridinium salts **12**. The pyrylium/pyridinium substituents were selected from a homologous set of aliphatic substituents of increased steric bulk, together with aromatic moieties that were proved to enhance binding in the active site of CA for sulfonamide inhibitors incorporating them.[30,31]

Thus, reaction of histamine **3** with substituted pyrylium salts of Chart 2, afforded a series of pyridinium derivatives of type **12**, as shown in Scheme 1.

We selected the ethylene spacer to be present in the molecules of the new CAAs reported here, of type **12**, due to the space restrictions in the active site of CA, ethylene being the shortest linker that can decouple the two heterocyclic moieties both

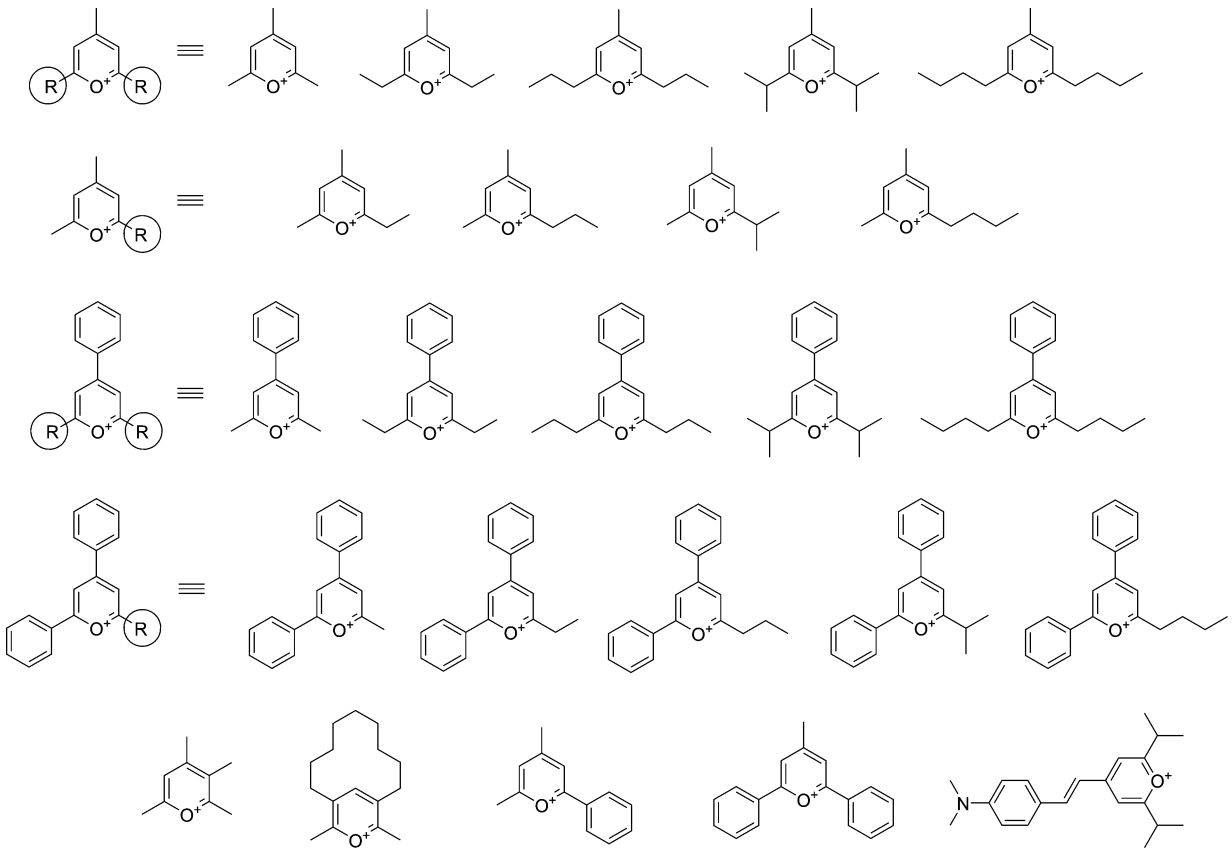
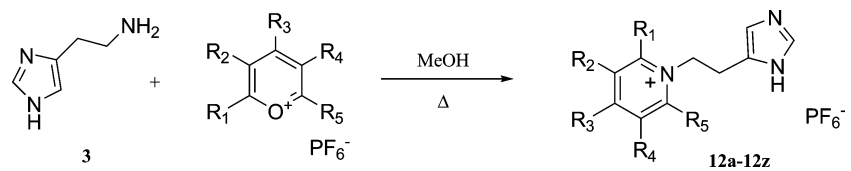


Chart 2 Pyrylium salts used in this study; small aliphatic (Me) or aromatic (phenyl) substituents were used in combination with a homologous aliphatic group set to sample various pockets of the active site of CA isozymes.



Scheme 1 Preparation of pyridinium histamine derivatives **12** by reaction of histamine **3** with substituted pyrylium salts of Chart 2, according to the Bayer–Piccard reaction.[46]

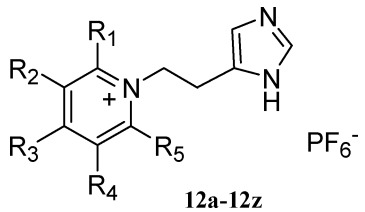
electronically and conformationally. Furthermore, this spacer offers a large degree of flexibility for the imidazole ring related to the pyridinium one, therefore allowing the sampling of various binding pockets at the rim of CA active site by the pyridinium anchor while keeping the imidazole ring in the proton shuttling path of the active site. The R1–R5 moieties substituting the pyridinium ring of compounds **12** encompass a large number of alkyl, aryl, and alkenyl moieties, starting with the compact methyl ones to the C2–C4, and ending with the much bulkier phenyl and substituted styryl moieties, as well as combination of various such variants (Table 1 and Chart 2). We have shown earlier that for sulfonamide CAIs incorporating substituted pyridinium moieties (such as the ones present in the activators **12**), the parameter which strongly influenced enzyme inhibition was just the nature of the groups substituting the pyridinium ring.[47–49] Indeed, an X-ray crystal structure of one such compound, the sulfonamide **13**,[50] in complex with CA II showed the trimethylpyridinium ring to bind effectively within the active site, towards its exit, and to make a strong, parallel π-stacking with the phenyl ring of Phe131, an amino acid residue critical for the binding of many classes of CAIs.[50] Thus, we expected that similarly to sulfonamide CAIs possessing the same moieties, the substituted pyridinium rings present in the histamine derivatives **12** will modulate the interaction between the enzyme and activator molecule, and thus afford interesting SAR insights for this new class of CAAs.

CA activation studies

Activation data of three physiologically relevant, cytosolic CA isozymes, the human (h) hCA I, II and VII with compounds **12** and histamine **3** as standard compound are shown in Table 1. The following SAR may be observed from the data of Table 1:

(i) Against the slow red cell isozyme hCA I, histamine **3** acts as a low micromolar activator,[29] with an activation constant of 2 μM, whereas its substituted pyridinium derivatives **12** show a range of activities, with K_As of 0.5 nM–93 μM. It is thus clear that the number and nature of moieties R1–R5 substituting the pyridinium ring present in compounds **12** are essential for the biological

Table 1 hCA I, II and VII activation with histamine pyridinium derivatives **12a–12z** and histamine, by a stopped-flow, CO_2 hydrase assay.[51]



12a-12z

						K_A* (μM)		
No.	R1	R2	R3	R4	R5	hCA I	hCA II	hCA VII
Histamine[a]						2	125	37.5
12a	Me	H	Ph	H	Me	58	4.76	2.15
12b	Et	H	Ph	H	Et	56	3.18	0.014
12c	*n*-Pr	H	Ph	H	*n*-Pr	0.95	23	19
12d	*i*-Pr	H	Ph	H	*i*-Pr	0.74	78	65
12e	*n*-Bu	H	Ph	H	*n*-Bu	4.53	21	23
12f	Me	H	Me	H	Me	3.15	18	7.71
12g	Et	H	Me	H	Et	0.0005	43	1.15
12h	*n*-Pr	H	Me	H	*n*-Pr	7.13	66	52
12i	*i*-Pr	H	Me	H	*i*-Pr	0.63	0.009	0.13
12j	*n*-Bu	H	Me	H	*n*-Bu	0.089	19	24
12k	Me	H	Me	H	Et	37	45	1.12
12m	Me	H	Me	H	*n*-Pr	43	31	1.16
12n	Me	H	Me	H	*i*-Pr	24	0.043	7.53
12o	Me	H	Me	H	*n*-Bu	7.88	0.13	8.16
12p	Me	Me	Me	H	Me	0.24	0.11	0.008
12q	Me	H	Ph	H	Ph	79	44	67
12r	Et	H	Ph	H	Ph	84	31	75
12s	*n*-Pr	H	Ph	H	Ph	93	0.018	81
12t	*i*-Pr	H	Ph	H	Ph	65	0.127	46
12u	*n*-Bu	H	Ph	H	Ph	60	51	74
12v	Ph	H	Me	H	Me	34	0.92	53
12x	Ph	H	Me	H	Ph	58	0.97	79
12y	Me	3,5-$(CH_2)_9$-			Me	29	1.15	0.073
12z	*i*-Pr	H	Me_2N-styryl	H	*i*-Pr	77	0.99	12.5

[a] From ref. 52

activity of these CAAs, similar to the situation already observed for sulfonamide CAIs possessing the same substitution pattern, *i.e.*, substituted pyridinium moieties attached to aromatic/heterocyclic sulfonamide scaffolds.[47–49] The moieties present in **12a**, **12b**, **12k–12n** and **12q–12z**, generally led to ineffective hCA I activators, as all these compounds showed activation constants higher than those of the parent, lead compound **3** (K_As of 24–93 μM). It may be observed that they incorporate either the compact 2,6-dimethyl- and 2,6-diethylpyridinium moieties (**12a** and **12b**), the 2,4-dimethyl-6-alkyl-pyridinium ones (**12k–12n**) or the bulkier moieties present in derivatives **12q–12z** (*i.e.*, 4,6-diphenyl-pyridinium-, 2-phenyl-substituted pyridinium-, 3,5-nonamethylene-2,6-dimethylpyridinium- or di-isopropyl-styrylpyridinium- groups). On the other hand, another group of derivatives, such as **12e**, **12f**, **12h** and **12o**, showed much better hCA I activatory properties compared to the compounds discussed above (but still inferior to histamine **3**), with K_As in the range of 3.15–7.88 μM. These compounds incorporate the 2,6-di-*n*-butyl, 2,4,6-trimethyl-, 2,6-di-*n*-propyl-4-methyl- and 2,4-dimethyl-6-*n*-butyl-pyridinium moieties in their molecules. However, excellent hCA I activating properties were observed for compounds **12c**, **12d**, **12g**, **12i**, **12j** and **12p**, which had K_As in the range of 0.5 nM–0.95 μM (Table 1). Thus, for the 2,6-disubstituted pyridinium salts investigated here, an enlargement of the substituents at the pyridinium ring from 2,6-dimethyl- to the corresponding di-isopropyl- or di-*n*-propyl-moieties, led to a significant enhancement of the enzyme activatory properties, of 61-times for **12c** compared to **12a**, and of 78-times for **12d** compared to **12a**, respectively. A dramatic increase has been observed by comparing **12b** with **12g**, which differ only by the presence of an additional 4-methyl group at the pyridinium ring of the last compound. These two compounds differ in hCA I activatory properties by a factor of 112 000. This is indeed remarkable both for the highly effective hCA I activating properties of **12g** on the one hand, but also because of the huge increase in activity induced by a small structural change (an additional CH_2 moiety), the highest one ever observed in a congener series of CAAs. Thus, these and the remaining data of Table 1 show that a small structural variation in the scaffold of compounds **12** leads to very different biological activity against this isozyme, with all types of activities evidenced among the 24 new derivatives investigated here.

(ii) The physiologically dominant cytosolic isoform hCA II is weakly activated by histamine **3** (K_A of 125 μM)[29] whereas all compounds **12** reported here acted as much better CAAs (Table 1), with activation constants in the range of 9 nM–78 μM. The pyridinium salts **12c–12h**, **12j–12m**, **12q**, **12r** and **12u** were

the least effective activators in the investigated series, with K_As in the range of 18–78 μM. They incorporate 2,6-disubstituted-pyridinium rings with bulkier alkyl moieties (**12c–12e**) or 2,4,6-trisubstituted- such moieties with C1–C4 alkyl groups (**12f–12h**, **12j–12m**). The diarylsubstituted pyridinium salts **12q**, **12r** and **12u** also belong to this subtype. However, the remaining derivatives, *i.e.*, **12a**, **12b**, **12i**, **12n–12p**, **12s**, **12t**, **12v–12z**, showed much more effective hCA II activating properties compared to their congeners, with low micromolar to low nanomolar activation constants (Table 1). Thus, again small structural changes in the structure of these derivatives led to drastic effects on their hCA II activating properties, as for the previous isoform discussed above. For example, compounds **12h** and **12i** are isomers, with the only difference being the presence of *n*-propyl or *iso*-propyl groups in positions 2,6 of the pyridinium ring. However, **12i** was over 7000 times a better hCA II activator (K_A of 9 nM) compared to its isomer **12h**. A similar situation has also been observed for other pairs of compounds, *e.g.*, **12r/12s**, which differ by a factor of about 1700 as hCA II activators. It is important to observe that for this isoform, unlike for hCA I discussed above, most of the compounds incorporating bulky, aromatic moieties (*e.g.* **12s**, **12t**, **12v–12z**) showed very good, submicromolar activation constants, being thus orders of magnitude better CAAs compared to the lead molecule **3**. Thus, SAR is rather different for these compounds when acting as hCA I and hCA II activators. For example, the best hCA I activator, compound **12g** (K_A of 0.5 nM), was only a medium efficiency hCA II activator (K_A of 43 μM).

(iii) The third cytosolic isoform investigated here, hCA VII, was more efficiently activated by histamine **3** than hCA II was, but less effectively than hCA I was (Table 1).[52] hCA VII is a much less investigated isoform compared to the previous two discussed above, and only recently has it been shown that it may be involved in the mediation of neuropathic pain, being thus a possible drug target.[53] Compared to hCA I and II which are ubiquitous in mammals,[1–3] hCA VII shows a more localized distribution, being however abundant in the vertebrate brain.[2,52,53] Data of Table 1 show that hCA VII was activated by all pyridinium salts **12** investigated here, but with a distinct profile compared to the other two isoforms. Thus, compounds **12c–12e**, **12j**, **12h** and **12q–12x** showed medium potency – weak hCA VII activating properties, with K_As in the range of 19–81 μM. The substitution patterns at the pyridinium ring present in these compounds include the 4-phenyl-2,6-disubstituted salts **12c–12d** incorporating bulkier alkyl moieties (*n*-Pr, *i*-Pr and *n*-Bu), the trialkyl-substituted derivatives **12h** and **12j**, as well as the compounds with one or two phenyl moieties **12q–12x**. Another group of compounds, among which are **12a**, **12f**, **12n**, **12o** and **12z**, showed better affinity for hCA VII, with activation constants in the range of 2.15–12.5 μM. They also have heterogeneous substitution patterns, such as the disubstituted one **12a**, the trisubstituted ones with only alkyl moieties **12f**, **12n** and **12o**, or the one incorporating the styryl moiety, **12z**. A third group of derivatives showed however excellent hCA VII activating properties, with K_As of 8 nM–1.16 μM. They include the diethylpyridinium derivative **12b**, the trisubstituted ones **12g**, **12i**, **12k** and **12m** (possessing only alkyl moieties at the pyridinium ring), and the tetrasubstituted pyridinium salts **12p** and **12y**. Again the nature of the moieties substituting the pyridinium ring and their position on it are the main factors influencing biological activity, as for hCA I and II. Small structural changes in these compounds led to dramatic effects on their hCA VII activating properties. For example, again the difference in activation constants for the pair **12h/12i** is very important, with the second compound being 400 times a better hCA VII activator compared to **12h**, although they are isomers and differ only by the presence of *n*-Pr *versus* *i*-Pr moieties in their molecules. The tetramethylpyridinium derivative **12p** was on the other hand a 963-times better hCA VII activator compared to the trimethyl-substituted one **12f**, of which it differs only by an additional CH_2 moiety. All these data clearly demonstrate that we are in the presence of a congener series of CAAs with very interesting biological activity,[54] ranging from highly effective, low nanomolar activators, to micromolar ones, being differentiated only by minor structural changes. Ongoing X-ray crystallography studies with activators of different potencies will shed more light towards the structural basis of these differences in activation efficiency.

Experimental section

General

Melting points were determined on a Thermolyne heating plate microscope and are uncorrected. The NMR spectra were recorded in DMSO-d_6, at ≈300 K with a Bruker Avance III spectrometer operating at 400 MHz for ^{1}H and at 100 MHz for ^{13}C. Chemical shifts are reported as δ values, using TMS as internal standard for proton spectra and the solvent resonance for carbon spectra. Coupling constants (*J*) are reported in Hz. Peak shapes were denoted as follows: s, singlet; d, doublet; t, triplet; q, quadruplet; sext, sextuplet; hept, heptuplet; m, multiplet; bs, broad singlet. Attributions were done by means of chemical shifts, peak integration, COSY, HMQC and HMBC experiments, and model spectra. HPLC-DAD-MS analysis (ESI) was done on an Agilent 1100 HPLC system equipped with a G1315A DAD and a 6130 Quadrupole MS. Combustion and HPLC confirmed the purity of all compounds to be over 95%. 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. Thin layer chromatography (TLC) was performed on silicagel 60-F_{254} plates (Merck, Whitehouse Station, NJ), eluted with MeOH:$CHCl_3$ 20:80 (v/v).

All reagents and solvents were purchased from Fisher Scientific (Pittsburgh, PA), VWR (West Chester, PA), or from Sigma-Aldrich (St. Louis, MO) and were used without further purification. Flash column chromatography was performed using Silicycle silica gel SiliaFlash R12030B grade 60 Å (40–63 μm). Pyrylium salts were prepared by the Balaban–Nenitzescu–Praill reaction and related procedures,[45] optimized in our laboratories. After rigorous purification, their structures were confirmed by ^{1}H-NMR and ^{13}C-NMR.

General procedure for the preparation of compounds 12

(Adapted from Dinculescu and Balaban.[55])

Histamine dihydrochloride (0.92 g, 5 mmol) was dissolved in minimum amount of water (200 μL) and was treated with a methanolic solution of sodium methoxide generated by reacting 115 mg Na metal with 10 mL of MeOH. The NaCl precipitate was filtered off and the methanolic filtrate containing the histamine

free base was added dropwise, under stirring, over a suspension of pyrylium hexafluorophosphate (6 mmol) in 10 mL MeOH. The homogenous mixture was refluxed for 5 min, and then was treated with 0.9 mL (15 mmol) glacial acetic acid and refluxed for another 1–2 h. Aqueous ammonium hydroxide 25% (0.5 mL) was added to the reaction mixture to convert any unreacted pyrylium salt into the corresponding pyridine. The solvent was evaporated to dryness and the residue was washed with ethyl ether, and then crystallized from MeOH or *i*PrOH. Advanced purification was achieved by column chromatography on SiO_2, using $MeOH/CH_2Cl_2$ gradient elution. Useful fractions were grouped, evaporated to dryness, and the purified product was crystallized from MeOH or *i*PrOH.

1-[2-(1*H*-Imidazol-4-yl)-ethyl]-2,6-dimethyl-4-phenylpyridinium hexafluorophosphate 12a. White crystals, mp = 299–306 °C, Yield 94%; ^{1}H-NMR (dmso-d^6), δ, ppm: 9.09 (s, 1H, NH Im), 8.40 (s, 2H, H-3,5 Py), 8.09 (m, 2H, H_{ortho} 4-Ph), 7.70 (m, 4H, H_{meta}, H_{para} 4-Ph and H-5 Im), 4.76 (t, J = 8.2 Hz, 2H, Py-$\underline{CH_2}$), 3.34 (t, J = 8.2 Hz, 2H, Im-$\underline{CH_2}$), 2.96 (s, 6H, $2CH_3$ α-Py); ^{13}C-NMR (dmso-d^6), δ, ppm: 156.6 (2C, C-2,6 Py), 154.7 (C-4 Py), 135.4 (4-Ph), 134.4 (C-2 Im), 133.0 (4-Ph), 130.6 (2C, 4-Ph), 129.3 (C-4 Im), 128.8 (2C, C-3,5 Py), 125.2 (2C, 4-Ph), 118.3 (C-5 Im), 51.3 (Py-$\underline{CH_2}$-), 23.5 (Im-$\underline{CH_2}$-), 20.8 (CH_3 γ-Py), 21.7 (2C, $2CH_3$ α-Py); Elemental analysis- Found: C, 51.35; H, 4.66; N 10.05%; M+, 278.1. $C_{18}H_{20}F_6N_3P$ requires C, 51.07; H, 4.76; N 9.93%; M+, 278.1.

1-[2-(1*H*-Imidazol-4-yl)-ethyl]-2,6-diethyl-4-phenylpyridinium hexafluorophosphate 12b. White crystals, mp = 269–272 °C, Yield 96%; ^{1}H-NMR (dmso-d^6), δ, ppm: 9.08 (s, 1H, NH Im), 8.26 (s, 2H, H-3,5 Py), 8.15 (m, 2H, H_{ortho} 4-Ph), 7.70 (m, 4H, H_{meta}, H_{para} 4-Ph and H-5 Im), 4.77 (t, J = 8.0 Hz, 2H, Py-$\underline{CH_2}$), 3.33 (t, J = 8.0 Hz, 2H, Im-$\underline{CH_2}$), 3.24 (q, J = 7.3 Hz, 4H, $2\underline{CH_2}CH_3$ α-Py), 1.48 (t, J = 7.3 Hz, 6H, $2CH_2\underline{CH_3}$ α-Py); ^{13}C-NMR (dmso-d^6), δ, ppm: 160.9 (2C, C-2,6 Py), 155.24 (C-4 Py), 135.3 (4-Ph), 134.7 (C-2 Im), 132.8 (C-4 Im), 130.4 (2C, 4-Ph), 129.0 (2C, C-3,5 Py), 123.5 (2C, 4-Ph), 118.3 (C-5 Im), 49.9 (Py-$\underline{C}H_2$-), 27.1 (2C, $2\underline{C}H_2CH_3$ α-Py), 24.9 (Im-$\underline{C}H_2$-), 13.9 (2C, $2CH_2\underline{C}H_3$ α-Py); Elemental analysis- Found: C, 53.48; H, 5.22; N 9.30%; M$^+$, 306.1. $C_{20}H_{24}F_6N_3P$ requires C, 53.22; H, 5.36; N 9.31%; M$^+$, 306.1.

1-[2-(1*H*-Imidazol-4-yl)-ethyl]-2,6-di-*n*-propyl-4-phenylpyridinium hexafluorophosphate 12c. White crystals, mp = 233–236 °C, Yield 82%; ^{1}H-NMR (dmso-d^6), δ, ppm: 9.03 (s, 1H, NH Im), 8.30 (s, 2H, H-3,5 Py), 8.13 (m, 2H, H_{ortho} 4-Ph), 7.70 (m, 3H, H_{meta}, H_{para} 4-Ph), 7.65 (s, 1H, H-5 Im), 4.78 (t, J = 7.7 Hz, 2H, Py-$\underline{CH_2}$), 3.32 (t, J = 7.7 Hz, 2H, Im-$\underline{CH_2}$), 3.14 (t, J = 7.7 Hz, 4H, $2\underline{CH_2}CH_2CH_3$ α-Py), 1.88 (sext, J = 7.3 Hz, 4H, $2CH_2\underline{CH_2}CH_3$ α-Py), 1.11 (t, J = 7.2 Hz, 6H, $2CH_2CH_2\underline{CH_3}$ α-Py); ^{13}C-NMR (dmso-d^6), δ, ppm: 159.7 (2C, C-2,6 Py), 154.8 (C-4 Py), 135.5 (4-Ph), 134.6 (C-2 Im), 133.0 (4-Ph), 130.5 (2C, 4-Ph), 129.4 (C-4 Im), 129.0 (2C, C-3,5 Py), 124.4 (2C, 4-Ph), 118.3 (C-5 Im), 50.2 (Py-$\underline{C}H_2$-), 35.4 (2C, $2\underline{C}H_2CH_2CH_3$ α-Py), 25.4 (Im-$\underline{C}H_2$-), 23.0 (2C, $2CH_2\underline{C}H_2CH_3$ α-Py), 14.5 (2C, $2CH_2CH_2\underline{C}H_3$ α-Py); Elemental analysis- Found: C, 55.41; H, 6.01; N 8.82%; M$^+$, 334.1. $C_{22}H_{28}F_6N_3P$ requires C, 55.11; H, 5.89; N 8.76%; M$^+$, 334.1.

1-[2-(1*H*-Imidazol-4-yl)-ethyl]-2,6-di-*iso*-propyl-4-phenylpyridinium hexafluorophosphate 12d. White crystals, mp = 190–193 °C, Yield 90%; ^{1}H-NMR (dmso-d^6), δ, ppm: 9.07 (s, 1H, NH Im), 8.28 (s, 2H, H-3,5 Py), 8.19 (m, 2H, H_{ortho} 4-Ph), 7.70 (m, 4H, H_{meta}, H_{para} 4-Ph and H-5 Im), 4.82 (t, J = 7.9 Hz, 2H, Py-$\underline{CH_2}$), 3.57 (hept, J = 6.6 Hz, 2H, $2\underline{CH}(CH_3)_2$ α-Py), 3.30 (t, J = 7.8 Hz, 2H, Im-$\underline{CH_2}$), 1.50 (d, J = 6.4 Hz, 12H, $2CH(\underline{CH_3})_2$ α-Py); ^{13}C-NMR (dmso-d^6), δ, ppm: 165.2 (2C, C-2,6 Py), 155.8 (C-4 Py), 135.4 (4-Ph), 134.9 (C-2 Im), 132.8 (4-Ph), 130.4 (2C, 4-Ph), 129.4 (2C, C-3,5 Py), 128.9 (C-4 Im), 121.8 (2C, 4-Ph), 118.5 (C-5 Im), 49.8 (Py-$\underline{C}H_2$-), 31.8 (2C, $2\underline{C}H(CH_3)_2$ α-Py), 26.1 (Im-$\underline{C}H_2$-), 23.5 (bs, 4C, $2CH(\underline{C}H_3)_2$ α-Py); Elemental analysis- Found: C, 55.32; H, 5.96; N 9.07%; M$^+$, 334.1. $C_{22}H_{28}F_6N_3P$ requires C, 55.11; H, 5.89; N 8.76%; M$^+$, 334.1.

1-[2-(1*H*-Imidazol-4-yl)-ethyl]-2,6-di-*n*-butyl-4-phenylpyridinium hexafluorophosphate 12e. White crystals, mp = 162–165 °C, Yield 42%; ^{1}H-NMR (dmso-d^6), δ, ppm: 8.70 (s, 1H, NH Im), 8.28 (s, 2H, H-3,5 Py), 8.14 (m, 2H, H_{ortho} 4-Ph), 7.70 (m, 3H, H_{meta}, H_{para} 4-Ph), 7.48 (s, 1H, H-5 Im), 4.77 (t, J = 7.8 Hz, 2H, Py-$\underline{CH_2}$), 3.29 (t, J = 7.7 Hz, 2H, Im-$\underline{CH_2}$), 3.14 (t, J = 7.5 Hz, 4H, $2\underline{CH_2}CH_2CH_2CH_3$ α-Py), 1.83 (m, 4H, $2CH_2\underline{CH_2}CH_2CH_3$ α-Py), 1.52 (sext, J = 7.2 Hz, 4H, $2CH_2CH_2\underline{CH_2}CH_3$ α-Py), 1.02 (t, J = 7.1 Hz, 6H, $2CH_2CH_2CH_2\underline{CH_3}$ α-Py); ^{13}C-NMR (dmso-d^6), δ, ppm: 159.0 (2C, C-2,6 Py), 153.9 (C-4 Py), 134.8 (C-2 Im), 133.7 (4-Ph), 132.0 (4-Ph), 129.8 (C-4 Im), 129.6 (2C, 4-Ph), 128.1 (2C, C-3,5 Py), 123.4 (2C, 4-Ph), 116.8 (C-5 Im), 49.8 (Py-$\underline{C}H_2$-), 32.6 (2C, $2\underline{C}H_2CH_2CH_2CH_3$ α-Py), 30.7 (2C, $2CH_2\underline{C}H_2CH_2CH_3$ α-Py), 25.3 (Im-$\underline{C}H_2$-), 22.0 (2C, $2CH_2CH_2\underline{C}H_2CH_3$ α-Py), 13.6 (2C, $2CH_2CH_2CH_2\underline{C}H_3$ α-Py); Elemental analysis- Found: C, 56.54; H, 6.65; N 8.56%; M$^+$, 362.1. $C_{24}H_{32}F_6N_3P$ requires C, 56.80; H, 6.36; N 8.28%; M$^+$, 362.1.

1-[2-(1*H*-Imidazol-4-yl)-ethyl]-2,4,6-trimethylpyridinium hexafluorophosphate 12f. White crystals, mp = 227–229 °C, Yield 33%; ^{1}H-NMR (dmso-d^6), δ, ppm: 9.14 (s, 1H, NH Im), 7.82 (s, 2H, H-3,5 Py), 7.68 (s, 1H, H-5 Im), 4.76 (t, J = 8.2 Hz, 2H, Py-$\underline{CH_2}$), 3.32 (t, J = 8.2 Hz, 2H, Im-$\underline{CH_2}$), 2.89 (s, 6H, $2CH_3$ α-Py), 2.54 (s, 3H, CH_3 γ-Py); ^{13}C-NMR (dmso-d^6), δ, ppm: 157.6 (C-4 Py), 154.6 (2C, C-2,6 Py), 134.0 (C-2 Im), 128.23 (C-4 Im), 128.20 (2C, C-3,5 Py), 117.3 (C-5 Im), 50.4 (Py-$\underline{C}H_2$-), 22.5 (Im-$\underline{C}H_2$-), 20.8 (CH_3 γ-Py), 20.6 (2C, $2CH_3$ α-Py); Elemental analysis- Found: C, 43.34; H, 5.38; N 11.47%; M$^+$, 216.1. $C_{13}H_{18}F_6N_3P$ requires C, 43.22; H, 5.02; N 11.63%; M$^+$, 216.1.

1-[2-(1*H*-Imidazol-4-yl)-ethyl]-2,6-diethyl-4-methylpyridinium hexafluorophosphate 12g. White crystals, mp = 238-241 °C, Yield 58%; ^{1}H-NMR (dmso-d^6), δ, ppm: 9.07 (s, 1H, NH Im), 7.80 (s, 2H, H-3,5 Py), 7.66 (s, 1H, H-5 Im), 4.70 (t, J = 8.2 Hz, 2H, Py-$\underline{CH_2}$), 3.27 (t, J = 8.2 Hz, 2H, Im-$\underline{CH_2}$), 3.14 (q, J = 7.3 Hz, 4H, $2\underline{CH_2}CH_3$ α-Py), 2.60 (s, 3H, CH_3 γ-Py), 1.39 (t, J = 7.3 Hz, 6H, $2CH_2\underline{CH_3}$ α-Py); ^{13}C-NMR (dmso-d^6), δ, ppm: 158.7 (2C, C-2,6 Py), 158.3 (C-4 Py), 134.5 (C-2 Im), 128.1 (C-4 Im), 126.1 (2C, C-3,5 Py), 117.4 (C-5 Im), 48.8 (Py-$\underline{C}H_2$-), 25.7 (2C, $2\underline{C}H_2CH_3$ α-Py), 23.7 (Im-$\underline{C}H_2$-), 21.2 (CH_3 γ-Py), 12.6 (2C, $2CH_2\underline{C}H_3$ α-Py); Elemental analysis- Found: C, 46.60; H, 5.48; N 11.05%; M$^+$, 244.1. $C_{15}H_{22}F_6N_3P$ requires C, 46.28; H, 5.70; N 10.79%; M$^+$, 244.1.

1-[2-(1*H*-Imidazol-4-yl)-ethyl]-2,6-di-*n*-propyl-4-methylpyridinium hexafluorophosphate 12h. White crystals, mp = 172–175 °C, Yield 83%; ^{1}H-NMR (dmso-d^6), δ, ppm: 9.08 (s, 1H, NH Im), 7.80 (s, 2H, H-3,5 Py), 7.64 (s, 1H, H-5 Im), 4.71 (t, J = 7.9 Hz, 2H, Py-$\underline{CH_2}$), 3.27 (t, J = 7.8 Hz, 2H, Im-$\underline{CH_2}$),

3.03 (t, J = 7.7 Hz, 4H, 2$\underline{CH_2}CH_2CH_3$ α-Py), 2.59 (s, 3H, CH_3 γ-Py), 1.79 (sext, J = 7.5 Hz, 4H, $2CH_2\underline{CH_2}CH_3$ α-Py), 1.06 (t, J = 7.3 Hz, 6H, $2CH_2CH_2\underline{CH_3}$ α-Py); ^{13}C-NMR (dmso-d^6), δ, ppm: 157.8 (C-4 Py), 157.5 (2C, C-2,6 Py), 134.5 (C-2 Im), 128.1 (C-4 Im), 127.1 (2C, C-3,5 Py), 117.4 (C-5 Im), 48.9 (Py-$\underline{C}H_2$-), 34.0 (2C, 2$\underline{C}H_2CH_2CH_3$ α-Py), 24.1 (Im-$\underline{C}H_2$-), 21.6 (2C, $2CH_2\underline{C}H_2CH_3$ α-Py), 21.1 (CH_3 γ-Py), 13.4 (2C, $2CH_2CH_2\underline{C}H_3$ α-Py); Elemental analysis- Found: C, 49.08; H, 6.40; N 9.93%; M$^+$, 272.1. $C_{17}H_{26}F_6N_3P$ requires C, 48.92; H, 6.28; N 10.07%; M$^+$, 272.1.

1-[2-(1*H*-Imidazol-4-yl)-ethyl]-2,6-di-*iso*-propyl-4-methylpyridinium hexafluorophosphate 12i. White crystals, mp = 214–217 °C, Yield 72%; ^{1}H-NMR (dmso-d^6), δ, ppm: 9.08 (s, 1H, NH Im), 7.92 (s, 2H, H-3,5 Py), 7.68 (s, 1H, H-5 Im), 4.76 (t, J = 7.9 Hz, 2H, Py-$\underline{CH_2}$), 3.49 (hept, J = 6.6 Hz, 2H, 2$\underline{CH}(CH_3)_2$ α-Py), 3.24 (t, J = 7.8 Hz, 2H, Im-$\underline{CH_2}$), 2.62 (s, 3H, CH_3 γ-Py), 1.40 (d, J = 6.5 Hz, 12H, $2CH(\underline{CH_3})_2$ α-Py); ^{13}C-NMR (dmso-d^6), δ, ppm: 163.8 (2C, C-2,6 Py), 159.5 (C-4 Py), 135.4 (C-2 Im), 128.8 (C-4 Im), 125.6 (2C, C-3,5 Py), 118.5 (C-5 Im), 49.4 (Py-$\underline{C}H_2$-), 31.3 (2C, 2$\underline{C}H(CH_3)_2$ α-Py), 26.1 (Im-$\underline{C}H_2$-), 23.5 (bs, 4C, $2CH(\underline{C}H_3)_2$ α-Py), 22.1 (CH_3 γ-Py); Elemental analysis- Found: C, 49.11; H, 6.44; N 10.01%; M$^+$, 272.1. $C_{17}H_{26}F_6N_3P$ requires C, 48.92; H, 6.28; N 10.07%; M$^+$, 272.1.

1-[2-(1*H*-Imidazol-4-yl)-ethyl]-2,6-di-*n*-butyl-4-methylpyridinium hexafluorophosphate 12j. White crystals, mp = 185–188 °C, Yield 34%; ^{1}H-NMR (dmso-d^6), δ, ppm: 9.08 (s, 1H, NH Im), 7.81 (s, 2H, H-3,5 Py), 7.63 (s, 1H, H-5 Im), 4.70 (t, J = 7.8 Hz, 2H, Py-$\underline{CH_2}$), 3.26 (t, J = 7.7 Hz, 2H, Im-$\underline{CH_2}$), 3.04 (t, J = 7.7 Hz, 4H, 2$\underline{CH_2}CH_2CH_2CH_3$ α-Py), 2.59 (s, 3H, CH_3 γ-Py), 1.74 (sext, J = 7.7 Hz, 4H, $2CH_2\underline{CH_2}CH_2CH_3$ α-Py), 1.48 (sext, J = 7.4 Hz, 4H, $2CH_2CH_2\underline{CH_2}CH_3$ α-Py), 0.99 (t, J = 7.3 Hz, 6H, $2CH_2CH_2CH_2\underline{CH_3}$ α-Py); ^{13}C-NMR (dmso-d^6), δ, ppm: 158.7 (2C, C-2,6 Py), 158.5 (C-4 Py), 135.4 (C-2 Im), 129.1 (C-4 Im), 127.9 (2C, C-3,5 Py), 118.2 (C-5 Im), 49.9 (Py-$\underline{C}H_2$-), 33.0 (2C, 2$\underline{C}H_2CH_2CH_2CH_3$ α-Py), 31.2 (2C, $2CH_2\underline{C}H_2CH_2CH_3$ α-Py), 25.0 (Im-$\underline{C}H_2$-), 22.7 (2C, $2CH_2CH_2\underline{C}H_2CH_3$ α-Py), 22.0 (CH_3 γ-Py), 14.5 (2C, $2CH_2CH_2CH_2\underline{C}H_3$ α-Py); Elemental analysis- Found: C, 50.96; H, 6.93; N 9.16%; M$^+$, 300.1. $C_{19}H_{30}F_6N_3P$ requires C, 51.23; H, 6.79; N 9.43%; M$^+$, 300.1.

1-[2-(1*H*-Imidazol-4-yl)-ethyl]-2-ethyl-4,6-dimethylpyridinium hexafluorophosphate 12k. White crystals, mp = 211–214 °C, Yield 51%; ^{1}H-NMR (dmso-d^6), δ, ppm: 9.06 (s, 1H, NH Im), 7.82 (s, 1H, H-5 Py), 7.80 (s, 1H, H-3 Py), 7.65 (s, 1H, H-5 Im), 4.69 (t, J = 8.2 Hz, 2H, Py-$\underline{CH_2}$), 3.27 (t, J = 8.2 Hz, 2H, Im-$\underline{CH_2}$), 3.15 (q, J = 7.2 Hz, 2H, $\underline{CH_2}CH_3$ α-Py), 2.86 (s, 3H, CH_3 α-Py), 2.61 (s, 3H, CH_3 γ-Py), 1.38 (t, J = 7.2 Hz, 3H, $CH_2\underline{CH_3}$ α-Py); ^{13}C-NMR (dmso-d^6), δ, ppm: 159.6 (C-6 Py), 158.9 (C-2 Py), 155.5 (C-4 Py), 135.4 (C-2 Im), 129.1 (2C, C-5 Py + C-4 Im), 127.1 (C-3 Py), 118.3 (C-5 Im), 50.4 (Py-$\underline{C}H_2$-), 26.5 ($\underline{C}H_2CH_3$ α-Py), 24.1 (Im-$\underline{C}H_2$-), 21.9 (CH_3 γ-Py), 21.5 (CH_3 α-Py), 13.6 ($CH_2\underline{C}H_3$ α-Py); Elemental analysis- Found: C, 44.92; H, 5.63; N 11.32%; M$^+$, 230.1. $C_{14}H_{20}F_6N_3P$ requires C, 44.80; H, 5.37; N 11.20%; M$^+$, 230.1.

1-[2-(1*H*-Imidazol-4-yl)-ethyl]-2-*n*-propyl-4,6-dimethylpyridinium hexafluorophosphate 12m. White crystals, mp = 193–197 °C, Yield 31%; ^{1}H-NMR (dmso-d^6), δ, ppm: 9.07 (s, 1H, NH Im), 7.81 (s, 1H, H-5 Py), 7.79 (s, 1H, H-3 Py), 7.65 (s, 1H, H-5 Im), 4.70 (t, J = 8.0 Hz, 2H, Py-$\underline{CH_2}$), 3.27 (t, J = 8.0 Hz, 2H, Im-$\underline{CH_2}$), 3.03 (t, J = 7.7 Hz, 2H, $\underline{CH_2}CH_2CH_3$ α-Py), 2.85 (s, 3H, CH_3 α-Py), 2.56 (s, 3H, CH_3 γ-Py), 1.77 (sext, J = 7.3 Hz, 2H, $CH_2\underline{CH_2}CH_3$ α-Py), 1.06 (t, J = 7.3 Hz, 3H, $CH_2CH_2\underline{CH_3}$ α-Py); ^{13}C-NMR (dmso-d^6), δ, ppm: 158.7 (C-6 Py), 158.3 (C-2 Py), 155.6 (C-4 Py), 135.4 (C-2 Im), 129.3 (C-4 Im), 129.1 (C-5 Py), 128.0 (C-3 Py), 118.3 (C-5 Im), 50.5 (Py-$\underline{C}H_2$-), 34.8 ($\underline{C}H_2CH_2CH_3$ α-Py), 24.3 (Im-$\underline{C}H_2$-), 22.6 ($CH_2\underline{C}H_2CH_3$ α-Py), 21.9 (CH_3 γ-Py), 21.6 (CH_3 α-Py), 14.3 ($CH_2CH_2\underline{C}H_3$ α-Py); Elemental analysis- Found: C, 46.45; H, 6.07; N 10.44%; M$^+$, 244.1. $C_{15}H_{22}F_6N_3P$ requires C, 46.28; H, 5.70; N 10.79%; M$^+$, 244.1.

1-[2-(1*H*-Imidazol-4-yl)-ethyl]-2-*iso*-propyl-4,6-dimethylpyridinium hexafluorophosphate 12n. White crystals, mp = 179–182 °C, Yield 81%; ^{1}H-NMR (dmso-d^6), δ, ppm: 9.06 (s, 1H, NH Im), 7.90 (s, 1H, H-5 Py), 7.80 (s, 1H, H-3 Py), 7.64 (s, 1H, H-5 Im), 4.73 (t, J = 7.8 Hz, 2H, Py-$\underline{CH_2}$), 3.50 (hept, J = 6.6 Hz, 1H, $\underline{CH}(CH_3)_2$ α-Py), 3.27 (t, J = 7.8 Hz, 2H, Im-$\underline{CH_2}$), 2.85 (s, 3H, CH_3 α-Py), 2.58 (s, 3H, CH_3 γ-Py), 1.37 (d, J = 6.5 Hz, 6H, $CH(\underline{CH_3})_2$ α-Py); ^{13}C-NMR (dmso-d^6), δ, ppm: 164.1 (C-6 Py), 159.1 (C-2 Py), 155.2 (C-4 Py), 135.4 (C-2 Im), 129.3 (C-4 Im), 129.1 (C-5 Py), 125.5 (C-3 Py), 118.5 (C-5 Im), 50.3 (Py-$\underline{C}H_2$-), 31.0 ($\underline{C}H(CH_3)_2$ α-Py), 24.8 (Im-$\underline{C}H_2$-), 23.4 (bs, 2C, $CH(\underline{C}H_3)_2$ α-Py), 22.0 (CH_3 γ-Py), 21.9 (CH_3 α-Py); Elemental analysis- Found: C, 46.39; H, 5.91; N 10.64%; M$^+$, 244.1. $C_{15}H_{22}F_6N_3P$ requires C, 46.28; H, 5.70; N 10.79%; M$^+$, 244.1.

1-[2-(1*H*-Imidazol-4-yl)-ethyl]-2-*n*-butyl-4,6-dimethylpyridinium hexafluorophosphate 12o. White crystals, mp = 167–170 °C, Yield 65%; ^{1}H-NMR (dmso-d^6), δ, ppm: 12.0 (bs, 1H, NH Im), 7.76 (s, 1H, H-5 Py), 7.74 (s, 1H, H-3 Py), 7.65 (s, 1H, H-5 Im), 4.68 (t, J = 7.2 Hz, 2H, Py-$\underline{CH_2}$), 3.08 (t, J = 7.2 Hz, 2H, Im-$\underline{CH_2}$), 3.01 (t, J = 7.7 Hz, 2H, $\underline{CH_2}CH_2CH_2CH_3$ α-Py), 2.79 (s, 3H, CH_3 α-Py), 2.54 (s, 3H, CH_3 γ-Py), 1.69 (sext, J = 7.4 Hz, 2H, $CH_2\underline{CH_2}CH_2CH_3$ α-Py), 1.46 (sext, J = 7.3 Hz, 2H, $CH_2CH_2\underline{CH_2}CH_3$ α-Py), 0.98 (t, J = 7.3 Hz, 3H, $CH_2CH_2CH_2\underline{CH_3}$ α-Py); ^{13}C-NMR (dmso-d^6), δ, ppm: 158.5 (C-6 Py), 158.1 (C-2 Py), 155.4 (C-4 Py), 136.4 (C-2 Im), 129.1 (C-4 Im), 129.0 (C-5 Py), 127.8 (C-3 Py), 117.0 (bs, C-5 Im), 52.2 (Py-$\underline{C}H_2$-), 32.8 ($\underline{C}H_2CH_2CH_2CH_3$ α-Py), 31.3 ($CH_2\underline{C}H_2CH_2CH_3$ α-Py), 27.7 (Im-$\underline{C}H_2$-), 22.7 ($CH_2CH_2\underline{C}H_2CH_3$ α-Py), 21.8 (CH_3 γ-Py), 21.5 (CH_3 α-Py); 14.5 ($CH_2CH_2CH_2\underline{C}H_3$ α-Py); Elemental analysis- Found: C, 47.98; H, 6.12; N 10.34%; M$^+$, 258.1. $C_{16}H_{24}F_6N_3P$ requires C, 47.64; H, 6.00; N 10.42%; M$^+$, 258.1.

1-[2-(1*H*-Imidazol-4-yl)-ethyl]-2,3,4,6-tetramethylpyridinium hexafluorophosphate 12p. White crystals, mp = 203–206 °C, Yield 78%; ^{1}H-NMR (dmso-d^6), δ, ppm: 9.03 (s, 1H, NH Im), 7.76 (s, 1H, H-5 Py), 7.62 (s, 1H, H-5 Im), 4.70 (t, J = 8.3 Hz, 2H, Py-$\underline{CH_2}$), 3.28 (t, J = 8.3 Hz, 2H, Im-$\underline{CH_2}$), 2.87 (s, 3H, 6-CH_3 α-Py), 2.83 (s, 3H, 2-CH_3 α-Py), 2.81 (s, 3H, CH_3 β-Py); 2.40 (s, 3H, CH_3 γ-Py); ^{13}C-NMR (dmso-d^6), δ, ppm: 157.6 (C-4 Py), 155.7 (C-6 Py), 153.9 (C-2 Py), 135.4 (C-2 Im), 129.2 (C-4 Im), 129.0 (C-5 Py), 128.0 (2C, C-3,5 Py), 118.3 (C-5 Im), 51.8 (Py-$\underline{C}H_2$-), 23.7 (Im-$\underline{C}H_2$-), 21.6 (CH_3 γ-Py), 21.4 (CH_3 β-Py), 18.5 (6-CH_3 α-Py), 16.6 (2-CH_3 α-Py); Elemental analysis- Found: C, 45.16; H, 5.43; N 11.06%; M$^+$, 230.1. $C_{14}H_{20}F_6N_3P$ requires C, 44.80; H, 5.37; N 11.20%; M$^+$, 230.1.

1-[2-(1*H*-Imidazol-4-yl)-ethyl]-2-methyl-4,6-diphenylpyridinium hexafluorophosphate 12q. White crystals, mp = 225–228 °C, Yield 51%; ^{1}H-NMR (dmso-d^6), δ, ppm: 8.90 (s, 1H, NH Im), 8.64 (bs, 1H, H-5 Py), 8.28 (bs, 1H, H-3 Py), 8.20 (m, 2H, H_{ortho} 4-Ph), 7.66 (m, 6H, H_{meta}, H_{para} 4,6-diPh), 7.57 (m, 2H, H_{ortho} 6-Ph), 7.23 (s, 1H, H-5 Im), 4.75 (t, J = 7.1 Hz, 2H, Py-$\underline{CH_2}$), 3.16 (t, J = 7.1 Hz, 2H, Im-$\underline{CH_2}$), 3.06 (s, 3H, CH_3 α-Py); ^{13}C-NMR (dmso-d^6), δ, ppm: 157.2 (C-6 Py), 156.8 (C-2 Py), 154.8 (C-4 Py), 135.4 (4-Ph), 134.1 (C-2 Im), 133.6, 133.2, 131.6, 130.6 (2C), 130.0 (2C), 129.7 (2C), 129.2 (2C), 129.0 (C-4 Im), 126.7 (C-5 Py), 125.6 (C-3 Py), 117.8 (C-5 Im), 52.3 (Py-$\underline{C}H_2$-), 24.5 (Im-$\underline{C}H_2$-), 22.0 (CH_3 α-Py); Elemental analysis- Found: C, 57.01; H, 4.80; N 8.45%; M$^+$, 340.1. $C_{23}H_{22}F_6N_3P$ requires C, 56.91; H, 4.57; N 8.66%; M$^+$, 340.1.

1-[2-(1*H*-Imidazol-4-yl)-ethyl]-2-ethyl-4,6-diphenylpyridinium hexafluorophosphate 12r. White crystals, mp = 234–238 °C, Yield 58%; ^{1}H-NMR (dmso-d^6), δ, ppm: 8.89 (s, 1H, NH Im), 8.51 (d, J = 2.0 Hz, 1H, H-5 Py), 8.28 (d, J = 2.0 Hz, 1H, H-3 Py), 8.23 (m, 2H, H_{ortho} 4-Ph), 7.67 (m, 8H, H_{meta}, H_{para} 4,6-diPh, H_{ortho} 6-Ph), 7.19 (s, 1H, H-5 Im), 4.76 (t, J = 7.2 Hz, 2H, Py-$\underline{CH_2}$), 3.32 (q, J = 7.3 Hz, 2H, $\underline{CH_2}CH_3$ α-Py), 3.15 (t, J = 7.2 Hz, 2H, Im-$\underline{CH_2}$), 1.57 (t, J = 7.3 Hz, 3H, $CH_2\underline{CH_3}$ α-Py); ^{13}C-NMR (dmso-d^6), δ, ppm: 161.5 (C-6 Py), 156.7 (C-2 Py), 155.2 (C-4 Py), 135.4 (4-Ph), 134.3 (C-2 Im), 133.8, 133.2, 131.6, 130.5 (2C), 129.9 (2C), 129.8 (2C), 129.3 (2C), 128.9 (C-4 Im), 125.7 (C-5 Py), 125.0 (C-3 Py), 117.7 (C-5 Im), 51.8 (Py-$\underline{C}H_2$-), 27.4 ($\underline{C}H_2CH_3$ α-Py), 25.2 (Im-$\underline{C}H_2$-), 14.1 ($CH_2\underline{C}H_3$ α-Py); Elemental analysis- Found: C, 58.06; H, 4.75; N 8.34%; M$^+$, 354.1. $C_{24}H_{24}F_6N_3P$ requires C, 57.72; H, 4.84; N 8.41%; M$^+$, 354.1.

1-[2-(1*H*-Imidazol-4-yl)-ethyl]-2-*n*-propyl-4,6-diphenylpyridinium hexafluorophosphate 12s. White crystals, mp = 132–135 °C, Yield 47%; ^{1}H-NMR (dmso-d^6), δ, ppm: 8.89 (s, 1H, NH Im), 8.51 (bs, 1H, H-5 Py), 8.28 (bs, 1H, H-3 Py), 8.23 (m, 2H, H_{ortho} 4-Ph), 7.69 (m, 8H, H_{meta}, H_{para} 4,6-diPh, H_{ortho} 6-Ph), 7.19 (s, 1H, H-5 Im), 4.73 (t, J = 7.1 Hz, 2H, Py-$\underline{CH_2}$), 3.21 (t, J = 7.7 Hz, 2H, $\underline{CH_2}CH_2CH_3$ α-Py), 3.02 (t, J = 7.1 Hz, 2H, Im-$\underline{CH_2}$), 1.97 (sext, J = 7.2 Hz, 2H, $CH_2\underline{CH_2}CH_3$ α-Py), 1.15 (t, J = 7.2 Hz, 3H, $CH_2CH_2\underline{CH_3}$ α-Py); ^{13}C-NMR (dmso-d^6), δ, ppm: 160.1 (C-6 Py), 156.8 (C-2 Py), 154.8 (C-4 Py), 135.8 (4-Ph), 134.2 (C-2 Im), 133.9, 133.1, 131.5, 130.5 (2C), 129.9 (4C), 129.3 (2C), 128.9 (C-4 Im), 125.7 (C-5 Py), 125.6 (C-3 Py), 116.8 (C-5 Im), 52.7 (Py-$\underline{C}H_2$-), 35.6 ($\underline{C}H_2CH_2CH_3$ α-Py), 26.8 (Im-$\underline{C}H_2$-), 23.0 ($CH_2\underline{C}H_2CH_3$ α-Py), 14.6 ($CH_2CH_2\underline{C}H_3$ α-Py); Elemental analysis- Found: C, 58.47; H, 5.17; N 8.48%; M$^+$, 368.1. $C_{25}H_{26}F_6N_3P$ requires C, 58.48; H, 5.10; N 8.18%; M$^+$, 368.1.

1-[2-(1*H*-Imidazol-4-yl)-ethyl]-2-*iso*-propyl-4,6-diphenylpyridinium hexafluorophosphate 12t. White crystals, mp = 145–149 °C, Yield 43%; ^{1}H-NMR (dmso-d^6), δ, ppm: 8.89 (s, 1H, NH Im), 8.53 (bs, 1H, H-5 Py), 8.25 (m, 3H, H-3 Py, H_{ortho} 4-Ph), 7.69 (m, 8H, H_{meta}, H_{para} 4,6-diPh, H_{ortho} 6-Ph), 7.19 (s, 1H, H-5 Im), 4.74 (t, J = 7.0 Hz, 2H, Py-$\underline{CH_2}$), 3.68 (hept, J = 6.6 Hz, 1H, $\underline{CH}(CH_3)_2$ α-Py), 3.15 (t, J = 7.0 Hz, 2H, Im-$\underline{CH_2}$), 1.58 (d, J = 6.6 Hz, 6H, $CH(\underline{CH_3})_2$ α-Py); ^{13}C-NMR (dmso-d^6), δ, ppm: 165.8 (C-6 Py), 156.5 (C-2 Py), 155.4 (C-4 Py), 135.4 (4-Ph), 134.3 (C-2 Im), 134.0, 133.1, 131.5, 130.4 (2C), 129.9 (2C), 129.8 (2C), 129.4 (2C), 129.0 (C-4 Im), 125.7 (C-5 Py), 123.0 (C-3 Py), 117.6 (C-5 Im), 51.9 (Py-$\underline{C}H_2$-), 31.8 ($\underline{C}H(CH_3)_2$ α-Py), 25.9 (Im-$\underline{C}H_2$-), 23.5 (2C, $CH(\underline{C}H_3)_2$ α-Py); Elemental analysis- Found: C, 58.58; H, 5.02; N 8.32%; M$^+$, 368.1. $C_{25}H_{26}F_6N_3P$ requires C, 58.48; H, 5.10; N 8.18%; M$^+$, 368.1.

1-[2-(1*H*-Imidazol-4-yl)-ethyl]-2-*n*-butyl-4,6-diphenylpyridinium hexafluorophosphate 12u. White crystals, mp = 142–145 °C, Yield 53%; ^{1}H-NMR (dmso-d^6), δ, ppm: 8.89 (s, 1H, NH Im), 8.53 (bs, 1H, H-5 Py), 8.25 (bs, 1H, H-3 Py), 8.21 (m, 2H, H_{ortho} 4-Ph), 7.67 (m, 8H, H_{meta}, H_{para} 4,6-diPh, H_{ortho} 6-Ph), 7.18 (s, 1H, H-5 Im), 4.78 (t, J = 7.0 Hz, 2H, Py-$\underline{CH_2}$), 3.25 (t, J = 7.9 Hz, 2H, $\underline{CH_2}CH_2CH_2CH_3$ α-Py), 3.15 (t, J = 7.0 Hz, 2H, Im-$\underline{CH_2}$), 1.94 (m, 2H, $CH_2\underline{CH_2}CH_2CH_3$ α-Py), 1.59 (sext, J = 7.4 Hz, 2H, $CH_2CH_2\underline{CH_2}CH_3$ α-Py), 1.06 (t, J = 7.3 Hz, 3H, $CH_2CH_2CH_2\underline{CH_3}$ α-Py); ^{13}C-NMR (dmso-d^6), δ, ppm: 160.4 (C-6 Py), 156.8 (C-2 Py), 155.0 (C-4 Py), 135.4 (4-Ph), 134.2 (C-2 Im), 133.8, 133.2, 131.6, 130.5 (2C), 129.9 (2C), 129.8 (2C), 129.3 (2C), 129.0 (C-4 Im), 125.8 (C-5 Py), 123.0 (C-3 Py), 117.7 (C-5 Im), 51.9 (Py-$\underline{C}H_2$-), 33.8 ($\underline{C}H_2CH_2CH_2CH_3$ α-Py), 31.8 ($CH_2\underline{C}H_2CH_2CH_3$ α-Py), 25.4 (Im-$\underline{C}H_2$-), 23.0 ($CH_2CH_2\underline{C}H_2CH_3$ α-Py), 14.6 ($CH_2CH_2CH_2\underline{C}H_3$ α-Py); Elemental analysis- Found: C, 59.47; H, 5.79; N 8.13%; M$^+$, 382.1. $C_{26}H_{28}F_6N_3P$ requires C, 59.20; H, 5.35; N 7.97%; M$^+$, 382.1.

1-[2-(1*H*-Imidazol-4-yl)-ethyl]-2,4-dimethyl-6-phenylpyridinium hexafluorophosphate 12v. White crystals, mp = 209–212 °C, Yield 43%; ^{1}H-NMR (dmso-d^6), δ, ppm: 8.75 (s, 1H, NH Im), 8.03 (s, 1H, H-5 Py), 7.78 (s, 1H, H-3 Py), 7.65 (m, 3H, 6-Ph), 7.51 (m, 2H, 6-Ph), 7.13 (s, 1H, H-5 Im), 4.69 (t, J = 7.0 Hz, 2H, Py-$\underline{CH_2}$), 3.09 (t, J = 7.0 Hz, 2H, Im-$\underline{CH_2}$), 2.94 (s, 3H, CH_3 α-Py), 2.61 (s, 3H, CH_3 γ-Py); ^{13}C-NMR (dmso-d^6), δ, ppm: 158.9 (C-4 Py), 155.9 (C-6 Py), 155.5 (C-2 Py), 135.3 (C-2 Im), 133.5, 131.5, 130.7, 129.9 (2C), 129.7 (all from 6-Ph), 129.5 (C-4 Im), 129.5 (2C, C-3,5 Py) 117.5 (C-5 Im), 52.4 (Py-$\underline{C}H_2$-), 24.8 (Im-$\underline{C}H_2$-), 21.8 (CH_3 γ-Py), 21.7 (CH_3 α-Py); Elemental analysis- Found: C, 51.40; H, 4.99; N 10.30%; M$^+$, 278.1. $C_{18}H_{20}F_6N_3P$ requires C, 51.07; H, 4.76; N 9.93%; M$^+$, 278.1.

1-[2-(1*H*-Imidazol-4-yl)-ethyl]-2,6-diphenyl-4-methylpyridinium hexafluorophosphate 12x. White crystals, mp = 170–175 °C, Yield 40%; ^{1}H-NMR (dmso-d^6), δ, ppm: 8.72 (s, 1H, NH Im), 8.03 (s, 2H, H-3,5 Py), 7.70 (m, 10H, 2,6-Ph), 7.93 (s, 1H, H-5 Im), 4.76 (t, J = 6.3 Hz, 2H, Py-$\underline{CH_2}$), 2.72 (t, J = 6.3 Hz, 2H, Im-$\underline{CH_2}$), 2.70 (s, 3H, CH_3 γ-Py); ^{13}C-NMR (dmso-d^6), δ, ppm: 159.9 (C-4 Py), 155.8 (2C, C-2,6 Py), 135.4 (C-2 Im), 133.5 (2C), 131.9 (2C), 131.4 (2C), 130.1 (4C), 129.8 (4C, 2C from 2,6-diPh + 2C-3,5 Py), 128.8 (C-4 Im), 117.5 (C-5 Im), 53.7 (Py-$\underline{C}H_2$-), 25.1 (Im-$\underline{C}H_2$-), 22.0 (CH_3 γ-Py); Elemental analysis- Found: C, 56.58; H, 4.91; N 8.77%; M$^+$, 340.1. $C_{23}H_{22}F_6N_3P$ requires C, 56.91; H, 4.57; N 8.66%; M$^+$, 340.1.

1-[2-(1*H*-Imidazol-4-yl)-ethyl]-2,6-dimethyl-3,5-(nonane-1,9-diyl)pyridinium hexafluorophosphate 12y. White crystals, mp = 229–234 °C, Yield 47%; ^{1}H-NMR (dmso-d^6), δ, ppm: 9.01 (s, 1H, NH Im), 8.49 (s, 1H, H-4 Py), 7.60 (s, 1H, H-5 Im), 4.84 (t, J = 8.0 Hz, 2H, Py-$\underline{CH_2}$), 3.27 (t, J = 8.0 Hz, 2H, Im-$\underline{CH_2}$), 2.98 (t, J = 6.1 Hz, 4H, 3,5-$\underline{CH_2}(CH_2)_7\underline{CH_2}$-), 2.81 (s, 6H, $2CH_3$ α-Py), 1.71 (m, 4H, 3,5-$CH_2\underline{CH_2}(CH_2)_5\underline{CH_2}CH_2$-), 1.14 (m, 6H, 3,5-$(CH_2)_9$-), 0.98 (m, 4H, 3,5-$(CH_2)_9$-); ^{13}C-NMR (dmso-d^6), δ, ppm: 153.0 (2C, C-2,6 Py), 148.2 (C-4 Py), 138.0 (2C, C-3,5 Py), 135.5 (C-2 Im), 129.3 (C-4 Im), 118.3 (C-5 Im), 52.5 (Py-$\underline{C}H_2$-), 32.4 (2C, 3,5-bisCH_2), 27.5, 25.8 (2C), 25.2 (2C), 25.1 (2C), 23.8 (Im-$\underline{C}H_2$-),

17.7 (2C, $2CH_3$ α-Py); Elemental analysis- Found: C, 53.68; H, 7.16; N 9.02%; M^+, 326.1. $C_{21}H_{32}F_6N_3P$ requires C, 53.50; H, 6.84; N 8.91%; M^+, 326.1.

4-[2-(4-Dimethylaminophenyl)-vinyl]-1-[2-(1*H*-imidazol-4-yl) ethyl]-2,6-di-isopropylpyridinium hexafluorophosphate 12z. Yellow crystals, mp = 201–204 °C, Yield 55%; ^{1}H-NMR (dmso-d^6), δ, ppm: 9.08 (s, 1H, NH Im), 8.07 (d, J = 16.1 Hz, 1H, CH = CH-Py), 7.97 (s, 2H, H-3,5 Py), 7.68 (s, 1H, H-5 Im), 7.65 (d, J = 8.9 Hz, 1H, H-2,6 Ph), 7.15 (d, J = 16.1 Hz, 1H, CH = CH-Py), 6.85 (d, J = 8.9 Hz, 1H, H-3,5 Ph), 4.69 (t, J = 7.7 Hz, 2H, Py-CH_2), 3.45 (hept, J = 6.6 Hz, 2H, $2CH(CH_3)_2$ α-Py), 3.24 (t, J = 7.6 Hz, 2H, Im-CH_2), 3.07 (s, 6H, $N(CH_3)_2$), 1.44 (d, J = 6.4 Hz, 12H, $2CH(CH_3)_2$ α-Py); ^{13}C-NMR (dmso-d^6), δ, ppm: 163.6 (2C, C-2,6 Py), 154.4 (C-4 Py), 152.8 (Ph), 142.8 (Ph), 135.4 (C-2 Im), 130.9 (2C, C-3,5 Py), 128.9 (C-4 Im), 123.59 (CH = CH-Py), 119.8 (2C, Ph), 118.5 (C-5 Im), 118.2 (CH=CH-Py), 112.9 (2C, Ph), 48.9 (Py-CH_2-), 40.6 (2C, $N(CH_3)_2$), 31.3 (2C, $2CH(CH_3)_2$ α-Py), 26.2 (Im-CH_2-), 23.6 (bs, 4C, $2CH(CH_3)_2$ α-Py); Elemental analysis- Found: C, 57.16; H, 6.58; N 10.13%; M^+, 403.1. $C_{26}H_{35}F_6N_4P$ requires C, 56.93; H, 6.43; N 10.21%; M^+, 403.1.

CA enzyme assay

An Applied Photophysics stopped-flow instrument was used for assaying the CA catalysed CO_2 hydration activity.[51] Phenol red (at a concentration of 0.2 mM) was used as indicator, working at the absorbance maximum of 557 nm, with 10 mM Hepes (pH 7.5) as buffer, 0.1 M Na_2SO_4 (for maintaining constant ionic strength), following the CA-catalyzed CO_2 hydration reaction for a period of 10 s at 25 °C. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and activation constants. For each activator at least six traces of the initial 5-10% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of activators (10 mM) were prepared in distilled-deionized water and dilutions up to 0.001 μM were done thereafter with distilled-deionized water. Activator and enzyme solutions were preincubated together for 15 min (standard assay at room temperature, or for prolonged periods of 24–72 h, at 4 °C) prior to assay, in order to allow for the formation of the E–A complex. The activation constant (K_A), defined similarly with the inhibition constant K_I,[31,32] can be obtained by considering the classical Michaelis–Menten equation (equation 4), which has been fitted by non-linear least squares by using PRISM 3:

$$v = v_{max}/\{1 + K_M/[S]\,(1 + [A]_f/K_A)\} \quad (4)$$

where $[A]_f$ is the free concentration of activator.

Working at substrate concentrations considerably lower than K_M ($[S] << K_M$), and considering that $[A]_f$ can be represented in the form of the total concentration of the enzyme ($[E]_t$) and activator ($[A]_t$), the obtained competitive steady-state equation for determining the activation constant is given by eqn 5:[31,32]

$$v = v_0.\, K_A/\{K_A + ([A]_t - 0.5\,\{([A]_t + [E]_t + K_A) - ([A]_t + [E]_t + K_A)^2 - 4[A]_t.[E]t)^{1/2}\}\} \quad (5)$$

where v_0 represents the initial velocity of the enzyme-catalyzed reaction in the absence of activator.[31–33]

Conclusion

We report here a series of positively-charged derivatives which has been prepared by reaction of histamine with a large series of substituted pyrylium salts. These pyridinium histamine derivatives were investigated as activators of three cytosolic isoforms, hCA I, II and VII. Activities from the subnanomolar to the micromolar range were detected for these compounds as activators of the three CAs. The substitution pattern at the pyridinium ring was the main factor influencing activity, and the three isoforms showed different requirements for good activity, both regarding the number of groups at the pyridinium ring, and their nature, among a large number of alkyl, aryl and styryl such moieties. We were successful in identifying nanomolar potent and selective activators for each isozyme (*e.g.* **12g** for CA I, **12s** for CA II, **12b** for CA VII) and also activators with a relatively good activity against all isozymes tested such as **12i** and **12p**, valuable tools for future physiology and pathology studies.

Abbreviation list

CA	carbonic anhydrase
CAI	carbonic anhydrase inhibitor
CAA	carbonic anhydrase activator
Im	imidazol
Py	pyridinium
SAR	structure–activity relationship

Acknowledgements

This research was financed by a grant of the 6th Framework Programme (FP) of the European Union (DeZnIT project), by a grant of the 7th FP of EU (Metoxia project), as well as by Temple University School of Pharmacy – Dean's Office. K. D. acknowledges the receipt of the TUSP Alumni Association Award.

References

1 C. T. Supuran, Carbonic Anhydrases as Drug Targets: General Presentation, in *Drug Design of Zinc-Enzyme Inhibitors. Functional, Structural, and Disease Applications*, ed. C. T. Supuran, J.-Y. Winum, Wiley, Hoboken, 2009; pp 13–38.

2 C. T. Supuran, Carbonic anhydrases: novel therapeutic applications for inhibitors and activators, *Nat. Rev. Drug Discovery*, 2008, **7**, 168–181; C. T. Supuran, Carbonic anhydrase inhibitors, *Bioorg. Med. Chem. Lett.*, 2010, **20**, 3467–3474.

3 V. M. Krishnamurthy, G. K. Kaufman, A. R. Urbach, I. Gitlin, K. L. Gudiksen, D. B. Weibel and G. M. Whitesides, Carbonic anhydrase as a model for biophysical and physical-organic studies of proteins and protein-ligand binding, *Chem. Rev.*, 2008, **108**(3), 946–1051.

4 V. Alterio, A. Di Fiore, K. D'Ambrosio, C. T. Supuran, G. De Simone, X-Ray crystallography of CA inhibitors and its importance in drug design, in *Drug Design of Zinc-Enzyme Inhibitors: Functional, Structural, and Disease Applications*, ed. C. T. Supuran, J. Y. Winum, Wiley, Hoboken, 2009, pp 73–138; T. Stams, D. W. Christianson, X-ray crystallographic studies of mammalian carbonic anhydrase isozymes, in *The Carbonic Anhydrases - New Horizons*, ed. W. R. Chegwidden, N. Carter, Y. Edwards, Birkhauser Verlag, Basel, Switzerland, 2000, pp 159–74.

5 C. T. Supuran, Carbonic anhydrases: Catalytic and inhibition mechanisms, distribution and physiological roles, in *Carbonic Anhydrase - Its Inhibitors and Activators*, C. T. Supuran, A. Scozzafava, J. Conway, Eds. ,CRC Press, Boca Raton, 2004, pp 1–23.

6 D. W. Christianson and C. A. Fierke, Carbonic anhydrase: Evolution of the zinc binding site by nature and by design, *Acc. Chem. Res.*, 1996, **29**(7), 331–339.
7 S. Lindskog and D. N. Silverman, The catalytic mechanism of mammalian carbonic anhydrases, *Exs*, 2000, (90), 175–95.
8 S. K. Nair and D. W. Christianson, Unexpected Ph-Dependent Conformation of His-64, the Proton Shuttle of Carbonic Anhydrase II, *J. Am. Chem. Soc.*, 1991, **113**(25), 9455–9458.
9 D. N. Silverman and R. McKenna, Solvent-mediated proton transfer in catalysis by carbonic anhydrase, *Acc. Chem. Res.*, 2007, **40**(8), 669–675.
10 J. Y. Winum, J.-L. Montero, A. Scozzafava, C. T. Supuran, Zinc Binding Functions in the Design of Carbonic Anhydrase Inhibitors, in *Drug Design of Zinc-Enzyme Inhibitors. Functional, Structural, and Disease Applications*, ed. C. T. Supuran, J.-Y. Winum, Wiley: Hoboken, 2009, pp 39–72.
11 C. T. Supuran, A. Casini, A. Scozzafava, Development of sulfonamide carbonic anhydrase inhibitors, in *Carbonic Anhydrase: Its Inhibitors and Activators*, ed. C. T. Supuran, A. Scozzafava, J. Conway, CRC Press, Boca Raton, 2004, pp 67–147.
12 M. A. Ilies, M. D. Banciu, Nonsulfonamide carbonic anhydrase inhibitors, in *Carbonic Anhydrase - Its Inhibitors and Activators*, ed. C. T. Supuran, A. Scozzafava, J. Conway, CRC Press, Boca Raton, 2004, pp 209–241.
13 M. Ilies, A. Scozzafava, C. T. Supuran, Carbonic anhydrase activators, in *Carbonic Anhydrase - Its inhibitors and activators*, ed. C. T. Supuran, A. Scozzafava, J. Conway, CRC Press, Boca Raton, 2004, pp 317–352.
14 C. Temperini, A. Scozzafava, C. T. Supuran, Drug Design Studies of Carbonic Anhydrase Activators, in *Drug Design of Zinc-Enzyme Inhibitors - Functional, Structural, and Disease Applications*, ed. C. T. Supuran, J.-Y. Winum, Wiley, Hoboken, 2009, pp 473–486.
15 M. Leiner and G. Leiner, The activators of carbonic acid anhydratase, *Naturwissenschaften*, 1941, **29**, 195–197.
16 C. Ho and J. M. Sturtevant, Activation of Bovine Carbonic Anhydrase by Ethylenediamine Tetraacetic Acid, *Biochem. Biophys. Res. Commun.*, 1960, **3**(1), 20–23.
17 I. Puscas, G. Buzas, P. Contrasiu and P. Suranyi, Direct Activation by Histamine of the Carbonic-Anhydrase in the Human Gastric-Mucosa, *Rev. Roum. Biochim.*, 1979, **16**(4), 317–320.
18 D. N. Silverman, C. Tu and G. C. Wynns, Proton transfer between hemoglobin and the carbonic anhydrase active site, *J. Biol. Chem.*, 1978, **253**(8), 2563–7.
19 D. N. Silverman, L. Backman and C. Tu, Role of hemoglobin in proton transfer to the active site of carbonic anhydrase, *J. Biol. Chem.*, 1979, **254**(8), 2588–91.
20 S. R. Paranawithana, C. K. Tu, P. J. Laipis and D. N. Silverman, Enhancement of the Catalytic Activity of Carbonic Anhydrase-III by Phosphates, *J. Biol. Chem.*, 1990, **265**(36), 22270–22274.
21 J. B. Shelton and W. R. Chegwidden, Activation of Carbonic Anhydrase-III by Phosphate, *Biochem. Soc. Trans.*, 1988, **16**(5), 853–854.
22 J. L. Parkes and P. S. Coleman, Enhancement of carbonic anhydrase activity by erythrocyte membranes, *Arch. Biochem. Biophys.*, 1989, **275**(2), 459–68.
23 C. Temperini, A. Scozzafava and C. T. Supuran, Carbonic anhydrase activation and the drug design, *Curr. Pharm. Des.*, 2008, **14**, 708–715.
24 C. T. Supuran, A. Scozzafava, Activation of carbonic anhydrase isozymes, in *The Carbonic Anhydrases - New Horizons*, ed. W. R. Chegwidden, N. Carter, Y. Edwards, Eds.; Birkhauser Verlag: Basel, Switzerland, 2000, pp. 197–219.
25 C. T. Supuran, A. Dinculescu and A. T. Balaban, Carbonic-Anhydrase Activators 5. CA II Activation by 2,4,6-Trisubstituted Pyridinium Cations with 1-(Omega-Aminoalkyl) Side-Chains, *Rev. Roum. Chim.*, 1993, **38**(3), 343–349.
26 C. T. Supuran, M. Barboiu, C. Luca, E. Pop, M. E. Brewster and A. Dinculescu, Carbonic anhydrase activators.14. Syntheses of mono and bis pyridinium salt derivatives of 2-amino-5-(2-aminoethyl)- and 2-amino-5-(3-aminopropyl)-1,3,4-thiadiazole and their interaction with isozyme II, *Eur. J. Med. Chem.*, 1996, **31**(7–8), 597–606.
27 C. T. Supuran, A. T. Balaban, P. Cabildo, R. M. Claramunt, J. L. Lavandera and J. Elguero, Carbonic anhydrase activators. VII. Isozyme, II activation by bisazolyl-methanes, -ethanes and related azoles, *Biol. Pharm. Bull.*, 1993, **16**(12), 1236–9.
28 C. T. Supuran, R. M. Claramunt, J. L. Lavandera and J. Elguero, Carbonic anhydrase activators. XV. A kinetic study of the interaction of bovine isozyme II with pyrazoles, bis- and tris-azolyl-methanes, *Biol. Pharm. Bull.*, 1996, **19**(11), 1417–22.
29 F. Briganti, S. Mangani, P. Orioli, A. Scozzafava, G. Vernaglione and C. T. Supuran, Carbonic anhydrase activators: X-ray crystallographic and spectroscopic investigations for the interaction of isozymes I and II with histamine, *Biochemistry*, 1997, **36**, 10384–92.
30 M. A. Ilies, M. D. Banciu, M. Ilies, F. Chiraleu, F. Briganti, A. Scozzafava and C. T. Supuran, 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, *Eur. J. Med. Chem.*, 1997, **32**, 911–918.
31 M. Ilies, M. D. Banciu, M. A. Ilies, A. Scozzafava, M. T. Caproiu and C. T. Supuran, Carbonic anhydrase activators: design of high affinity isozymes I, II, and IV activators, incorporating tri-/tetrasubstituted-pyridinium-azole moieties, *J. Med. Chem.*, 2002, **45**(2), 504–10.
32 C. T. Supuran and A. Scozzafava, Carbonic anhydrase activators: amino acyl/dipeptidyl histamine derivatives bind with high affinity to isozymes I, II and IV and act as efficient activators, *Bioorg. Med. Chem.*, 1999, **7**(12), 2915–23.
33 A. Scozzafava and C. T. Supuran, Carbonic anhydrase activators: high affinity isozymes I, II, and IV activators, incorporating a beta-alanyl-histidine scaffold, *J. Med. Chem.*, 2002, **45**(2), 284–91.
34 F. Briganti, A. Scozzafava and C. T. Supuran, Novel carbonic anhydrase isozymes I, II and IV activators incorporating sulfonyl-histamino moieties, *Bioorg. Med. Chem. Lett.*, 1999, **9**(14), 2043–8.
35 C. T. Supuran and A. Scozzafava, Carbonic anhydrase activators: synthesis of high affinity isozymes I, II and IV activators, derivatives of 4-(arylsulfonylureido-amino acyl)ethyl-1H-imidazole, *J. Enzyme Inhib. Med. Chem.*, 2000, **15**(5), 471–86.
36 D. Duda, C. Tu, M. Qian, P. Laipis, M. Agbandje-McKenna, D. N. Silverman and R. McKenna, Structural and kinetic analysis of the chemical rescue of the proton transfer function of carbonic anhydrase II, *Biochemistry*, 2001, **40**, 1741–8.
37 I. Elder, S. Han, C. Tu, H. Steele, P. J. Laipis, R. E. Viola and D. N. Silverman, Activation of carbonic anhydrase II by active-site incorporation of histidine analogs, *Arch. Biochem. Biophys.*, 2004, **421**, 283–9.
38 I. Elder, C. Tu, L. J. Ming, R. McKenna and D. N. Silverman, Proton transfer from exogenous donors in catalysis by human carbonic anhydrase II, *Arch. Biochem. Biophys.*, 2005, **437**, 106–14.
39 C. Temperini, A. Scozzafava, L. Puccetti and C. T. Supuran, Carbonic anhydrase activators: X-ray crystal structure of the adduct of human isozyme II with L-histidine as a platform for the design of stronger activators, *Bioorg. Med. Chem. Lett.*, 2005, **15**, 5136–5141; C. Temperini, A. Scozzafava and C. T. Supuran, Carbonic anhydrase activators: The first X-ray crystallographic study of an adduct of isoform I, *Bioorg. Med. Chem. Lett.*, 2006, **16**, 5152–5156.
40 C. Temperini, A. Scozzafava, D. Vullo and C. T. Supuran, Carbonic anhydrase activators. Activation of isozymes I, II, IV, VA, VII, and XIV with l- and d-histidine and crystallographic analysis of their adducts with isoform II: engineering proton-transfer processes within the active site of an enzyme, *Chem.–Eur. J.*, 2006, **12**, 7057–7066; C. Temperini, D. Vullo, A. Scozzafava and C. T. Supuran, Carbonic anhydrase activators. Activation of isoforms I, II, IV, VA, VII and XIV with L- and D- phenylalanine and crystallographic analysis of their adducts with isozyme II: sterospecific recognition within the active site of an enzyme and its consequences for the drug design, *J. Med. Chem.*, 2006, **49**, 3019–3027.
41 A. Scozzafava and C. T. Supuran, Carbonic anhydrase activators. Part 24. High affinity isozymes I, II and IV activators, derivatives of 4-(4-chlorophenylsulfonylureido-amino acyl)ethyl-1H-imidazole, *Eur. J. Pharm. Sci.*, 2000, **10**, 29–41.
42 A. Scozzafava and C. T. Supuran, Carbonic anhydrase activators - Part 21. Novel activators of isozymes I, II and IV incorporating carboxamido and ureido histamine moieties, *Eur. J. Med. Chem.*, 2000, **35**, 31–9.
43 A. Scozzafava, B. Iorga and C. T. Supuran, Carbonic anhydrase activators: synthesis of high affinity isozymes I, II and IV activators, derivatives of 4-(4-tosylureido-amino acyl)ethyl-1H-imidazole (histamine derivatives), *J. Enzyme Inhib. Med. Chem.*, 2000, **15**, 139–61.
44 A. T. Balaban, C. Uncuta, A. Dinculescu, M. Elian and F. Chiraleu, Rotation barriers in N-substituted 2,4,6-trimethylpyridinium cations, *Tetrahedron Lett.*, 1980, **21**, 1553–1556; M. Ilies and M. T. Caproiu, Dynamic 1H-NMR Conformational Study in a Series of Pyridinium Pyrazoles, *Revista de Chimie*, 2007, **58**, 442–446.

45 A. T. Balaban and C. D. Nenitzescu, Pyrylium salts obtained by diacylation of olefins. II. The, two pyrylium salts formed in the diacylation of 2-methyl-2-butene, *J. Chem. Soc.*, 1961, 3553–3560; P. F. G. Praill and A. R. Whitear, Pyrylium salts and related compounds. Part I. The, reaction between olefins and acylium perchlorates, *J. Chem. Soc.*, 1961, 3573–3579; T. S. Balaban, A. T. Balaban, Pyrylium Salts, in *Science of Synthesis. Houben-Weyl Methods of Molecular Transformations*, Georg Thieme Verlag: Stuttgart, 2003; Vol. 14, pp 11–200.
46 A. Baeyer and J. Piccard, Untersuchungen uber das Dimethylpyron, *Justus Liebigs Ann. Chem.*, 1911, **384**, 208–224; A. Baeyer, J. Piccard and W. Gruber, Untersuchungen uber das Dimethylpyron, *Justus Liebigs Ann. Chem.*, 1915, **407**, 332–369; Z. Yoshida, H. Sugimoto, T. Sugimoto and S. Yoneda, Reaction of Thiopyrilium Cations with Amines, *J. Org. Chem.*, 1973, **38**(23), 3990–3993.
47 C. T. Supuran and B. W. Clare, Carbonic anhydrase inhibitors. Part 24. A quantitative structure-activity study of positively-charged sulfonamide inhibitors, *Eur. J. Med. Chem.*, 1995, **30**, 687–696; C. T. Supuran, A. Scozzafava, M. A. Ilies, B. Iorga, T. Cristea, F. Chiraleu and M. D. Banciu, Carbonic anhydrase inhibitors. Part 53. Synthesis of substituted-pyridinium derivatives of aromatic sulfonamides: the first non-polymeric membrane-impermeable inhibitors with selectivity for isozyme IV, *Eur. J. Med. Chem.*, 1998, **33**, 577–594.
48 C. T. Supuran, A. Scozzafava, M. A. Ilies and F. Briganti, Carbonic anhydrase inhibitors. Synthesis of sulfonamides incorporating 2,4,6-trisubstituted-pyridinium-ethylcarboxamido moieties possessing membrane-impermeability and *in vivo* selectivity for the membrane-bound (CA IV) *versus* the cytosolic (CA I and CA II) isozymes, *J. Enzyme Inhib. Med. Chem.*, 2000, **15**, 381–401; A. Scozzafava, F. Briganti, M. A. Ilies and C. T. Supuran, Carbonic anhydrase inhibitors: Synthesis of membrane-impermeant low molecular weight sulfonamides possessing *in vivo* selectivity for the membrane-bound *versus* the cytosolic isozymes, *J. Med. Chem.*, 2000, **43**, 292–300.
49 Ö. Güzel, A. Maresca, A. Scozzafava, A. Salman, A. T. Balaban and C. T. Supuran, Carbonic anhydrase inhibitors. Synthesis of 2,4,6-trimethylpyridinium derivatives of 2-(hydrazinocarbonyl)-3-aryl-1*H*-indole-5-sulfonamides acting as potent inhibitors of the tumor-associated isoform IX and XII, *Bioorg. Med. Chem. Lett.*, 2009, **19**, 2931–2934; J. R. Casey, P. E. Morgan, D. Vullo, A. Scozzafava, A. Mastrolorenzo and C. T. Supuran, Carbonic anhydrase inhibitors. Design of selective, membrane-impermeant inhibitors targeting the human tumor-associated isozyme IX, *J. Med. Chem.*, 2004, **47**, 2337–2347.
50 V. Menchise, G. De Simone, V. Alterio, A. Di Fiore, C. Pedone, A. Scozzafava and C. T. Supuran, Carbonic anhydrase inhibitors: Stacking with Phe131 determines active site binding region of inhibitors as exemplified by the X-ray crystal structure of a membrane-impermeant antitumor sulfonamide complexed with isozyme II, *J. Med. Chem.*, 2005, **48**, 5721–5727; M. Barboiu, C. T. Supuran, L. Menabuoni, A. Scozzafava, F. Mincione, F. Briganti and G. Mincione, Carbonic anhydrase inhibitors. Part 75. Synthesis of topically effective intraocular pressure lowering agents derived from 5-(ω-aminoalkylcarboxamido)-1,3,4-thiadiazole-2-sulfonamide, *J. Enz. Inhib.*, 1999, **15**, 23–46.
51 R. G. Khalifah, The carbon dioxide hydration activity of carbonic anhydrase. I. Stop-flow, kinetic studies on the native human isoenzymes B and C, *J. Biol. Chem.*, 1971, **246**, 2561–2573.
52 D. Vullo, A. Innocenti, I. Nishimori, A. Scozzafava, K. Kaila and C. T. Supuran, Carbonic anhydrase activators. Activation of the human isoforms VII (cytosolic) and XIV (transmembrane) with amino acids and amines, *Bioorg. Med. Chem. Lett.*, 2007, **17**, 4107–4112.
53 M. Asiedu, M. H. Ossipov, K. Kaila and T. J. Price, Acetazolamide and midazolam act synergistically to inhibit neuropathic pain, *Pain*, 2010, **148**, 302–308.
54 K. Dave, M. A. Ilies, A. Scozzafava, C. Temperini, D. Vullo and C. T. Supuran, An inhibitor-like binding mode of a carbonic anhydrase activator within the active site of isoform II, *Bioorg. Med. Chem. Lett.*, 2011, **21** , in press (PMID: 21036610).
55 A. Dinculescu and A. T. Balaban, Reactions of pyrylium salts with nucleophiles. XIV. New, pyridinium salts with potential biological activity, *Rev. Roum. Chim.*, 1980, **25**, 1505–1528; A. T. Balaban, A. Dinculescu, G. N. Dorofeenko, G. W. Fischer, A. V. Koblik, V. V. Mezheritskii, W. Schroth, Pyrylium Salts. Syntheses, reactions and Physical Properties, in *Advances in Heterocyclic Chemistry*, ed. A. R. Katritzky, Academic Press, New York, 1982, Vol. 2, p. 298.

