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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

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Abstract – Reaction of three aromatic sulfonamides containing a free amino group, i.e., sulfanilamide, homosulfanilamide and 4-(2-aminoethyl)-benzenesulfonamide with di-, tri- or tetra-substituted pyrylium salts afforded three series of cationic sulfonamides, containing a large variety of moieties substituting the pyridinium ring. The new derivatives were assayed as inhibitors of three carbonic anhydrase (CA) isozymes, CA I, II (cytosolic forms) and IV (membrane-bound form). Efficient inhibition was observed against all three isozymes, but due to the cationic nature of these inhibitors, in vivo and ex vivo experiments showed that only CA IV is selectively inhibited to a high degree, without affecting the cytosolic isozymes, present in appreciable concentrations in the experimental model used. This is the first example of selective in vivo inhibition of only one physiologically relevant CA isozyme with non-polymeric inhibitors and might lead to more selective drugs from this class of pharmacological agents. © Elsevier, Paris

carbonic anhydrase / sulfonamide / isozyme I, II, IV / pyridinium / membrane-impermeable / selective inhibition

1. Introduction

Carbonic anhydrase (CA, EC 4.2.1.1), an enzyme playing a central role to both transport and metabolic processes involving CO_2 and bicarbonate, is present in a variety of tissues of higher vertebrates in the form of eight isozymes [2–4]. By catalyzing the reversible interconversion between the two chemical species mentioned above, in metabolically active tissues (such as the muscle), cytoplasmic (CA I–III) and sarcolemmal (CA III) isozymes facilitate CO_2 transport out of the cell [3]. The only membrane-bound isozyme known (CA IV), which is highly abundant in the kidneys and lungs, has been shown to possess an extracellular orientation of the active site, and to be critical in acidifying the outer boundary layer through the protons formed by CO_2 hydration according to the following equation [5, 6]:

$$O{=}C{=}O + H_2O \Leftrightarrow HCO_3^- + H^+$$

This process further facilitates cellular ammonia transport by providing the H^+ ion for the protonation of NH_3, thus maintaining the trans-membrane ammonia gradient [3, 5]:

$$NH_3 + H^+ \Leftrightarrow NH_4^+$$

The mitochondrial isozyme (CA V) is known to supply bicarbonate/CO_2 for the initial reaction of gluconeogenesis and ureagenesis in many mammalian tissues [7, 8], as well as for the pyruvate carboxylation in the de novo lipogenesis in adipocytes (*figure 1*) [9].

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†For part 52, see [1]

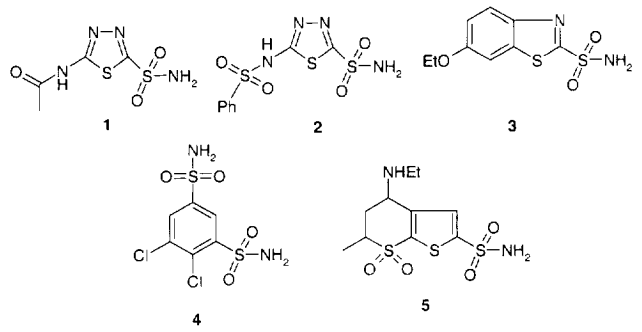


Figure 1.

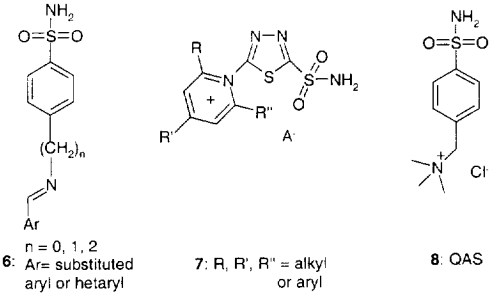


Figure 2.

Aromatic/heterocyclic sulfonamides with the general formula R-SO_2NH_2 are powerful inhibitors of CAs [4, 10–16], but relevant differences of affinity of these inhibitors for the different isozymes have been evidenced [4]. Thus, CA II is the most susceptible to inhibition by sulfonamides [4], followed by CA IV and V [6, 9], whereas CA I has generally a lower affinity for this type of inhibitors and a much larger one for the inorganic complexing anions, such as cyanide, cyanate, thiocyanate [4, 17–19]. Finally, CA III is a sulfonamide-resistant isozyme [20], being appreciably inhibited only at millimolar concentrations of inhibitor, whereas the other isozymes are inhibited at micromolar-nanomolar concentrations of sulfonamides such as acetazolamide **1**, benzolamide **2**, ethoxzolamide **3**, dichlorophenamide **4** or dorzolamide **5** – all clinically used drugs [21–24]. The main applications of such agents is as antiglaucoma drugs [22–24], but they are also used as antiulcer [21], diuretic [25], or antiepileptic drugs [26], as well as diagnostic tools in NMR imaging [27, 28]. Although many sulfonamide CA inhibitors possess high affinity for the major isozymes considered to play important physiological functions (such as CA II, CA IV and CA V) [4, 7, 8, 11–16], the critical challenge for the design of novel pharmacological agents from this class, is constituted by the lack of specificity of such compounds towards the different isozymes [4, 29, 30]. Among the eight isozymes described up to now, just CA II and CA IV have the most similar affinities to diverse sulfonamide inhibitors, although, as mentioned above, small differences between them exist, with CA II showing a larger affinity than CA IV for the largest majority of such compounds [4, 31–33]. This fact, as well as the physiological importance of these two isozymes, prompted much research in our laboratory in order to find compounds which might discriminate between CA II and CA IV [11, 12, 16, 29]. Thus, some Schiff bases of type **6** showed for the first time affinities comparable or slightly better towards CA IV as compared to CA II, but such compounds appreciably inhibited both isozymes [11, 12, 16]. Another approach proposed by us [13, 29, 34] consisted in preparing positively-charged sulfonamides of type **7**, which due to their cationic nature would be membrane-impermeable, and would inhibit only the enzyme located on the outer surface of the membranes, and not the cytosolic isozymes (when administered in vivo, of course). Indeed, Henry [35] has reported interesting physiological studies in the crab *Callinectes sapidus*, with a cationic sulfonamide of type **8**, QAS (quaternary ammonium sulfonamide), which allowed to determine the localization of different CA isozymes in the endolymph of this arthropode. Due to its cationic nature, QAS was unable to penetrate through biological membranes and inhibited only the membrane-bound CA, leaving the cytosolic isozymes unaffected (*figure 2*) [35].

In this paper we report the synthesis of a large series of cationic sulfonamides, prepared from aromatic sulfonamides of type **9**, possessing a free amino group in their molecule, and 2,6-di-, 2,4,6-tri- and 2,3,4,6-tetra-substituted pyrylium salts **10** (*figure 3*).

The cationic pyridinium derivatives **11–54** obtained in this way were expected to be unable to penetrate through biological membranes, as subsequently confirmed by ex

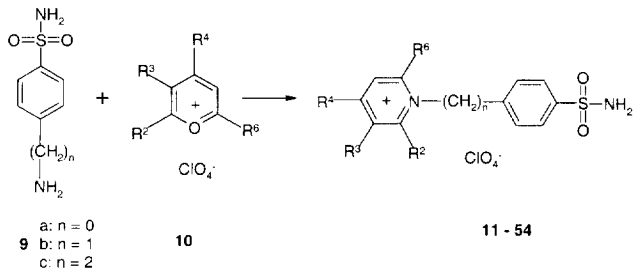


Figure 3.

vivo and in vivo experiments reported here. Although the new sulfonamides reported by us generally strongly inhibited in vitro both CA II and CA IV, in vivo, due to their cationic nature, the cytosolic isozymes were unaffected, whereas membrane-associated CA (isozyme IV) was strongly inhibited. This is the first study in which the selective inhibition of only one physiologically relevant CA isozyme is achieved with a low molecular weight type CA inhibitor and might lead to more selective drugs from this class of pharmacological agents.

2. Chemistry

The reaction between pyrylium salts and amines, leading to pyridinium salts, has been discovered by Baeyer and Piccard at the beginning of this century, and applied thereafter for the preparation of a large number of such derivatives, unaccessible by other synthetic procedures [36, 37].

Although this reaction has been widely used for the preparation of a variety of biologically active derivatives [38–43], in the sulfonamide series only few studies have been published. Thus, Neidlein and Witerzens [44] mentioned the 2,4,6-trimethylpyridinium derivative of sulfanilamide **11** in their paper, but no melting point or any other characterization of this compound followed. Dinculescu and Balaban [39] on the other hand reported and thoroughly characterized three pyridinium salts derived from homosulfanilamide **9b**, i.e., the 2,4,6-trimethyl **23**, 2-methyl-4,6-diphenyl **30** and 2,4,6-triphenylpyridinium **36** derivatives, respectively. Together with the large series of 2,4,6-trisubstituted 1-(2-sulfonamido-1,3,4-thiadiazol-5-yl)-pyridinium salts **7** reported by this group [13, 29], these are the only studies in which the interaction of pyrylium salts with sulfonamides has been investigated. Taking into account the remarkable biological activity of some of the above mentioned compounds, of type **7** [13, 29], it appeared of great interest to prepare a large series of pyridinium derivatives, containing the benzenesulfonamide moiety in their molecule.

Reaction of sulfanilamide **9a**, homosulfanilamide **9b** or 4-(2-aminoethyl)-benzenesulfonamide **9c** with 2,6-di-, 2,4,6-tri- or 2,3,4,6-tetrasubstituted pyrylium salts **10** afforded the pyridinium salts **11–54**, by the general Baeyer-Piccard synthesis [36, 37] (*figure 3*).

Compounds **11–54** have been characterized by analytic and spectroscopic methods that confirmed their structure (see Experimental protocols for details).

3. Pharmacology

Inhibition of three purified CA isozymes, hCA I, hCA II and bCA IV (h = human, b = bovine isozyme)

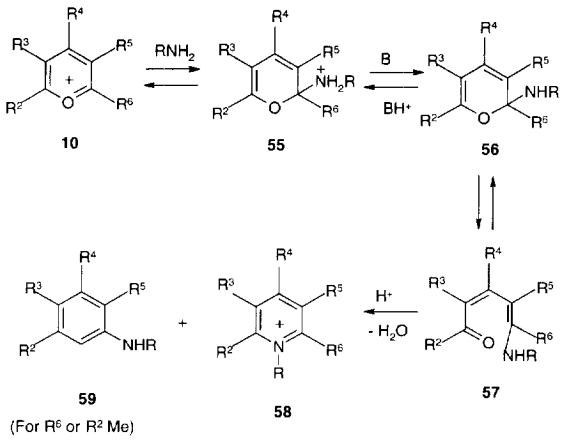


Figure 4.

with the new pyridinium salts **11–54** is shown in *table I*, whereas inhibition with standard inhibitors **1–5** as well as the starting sulfonamides **9a–c** is presented in *table II*.

Studies of penetrability through red cell membranes (ex vivo) with standard sulfonamide CA inhibitors and with some of the cationic sulfonamides synthesized by us are shown in *table III*, whereas *table IV* presents in vivo urine bicarbonate excretion studies in rat, after i.v. perfusion of classical (acetazolamide and benzolamide) and positively-charged inhibitors, together with the levels of sulfonamides present in red cells after treatment with these compounds.

4. Results and discussion

The apparently simple reaction between a pyrylium salt **10** and an amine, leading to pyridinium salts, is in reality a complicated process (*figure 4*), as established by detailed spectroscopic and kinetic data from Balaban's and Katritzky's groups [38, 39, 45–47].

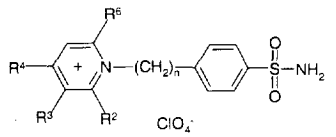
Thus, the nucleophilic attack of a primary amine RNH_2 on pyrylium cations generally occurs in the α position, with the formation of intermediates of type **55**, which by deprotonation in the presence of bases lead to the 2-amino-tetradehydropyran derivatives **56**. In many cases the deprotonation reaction is promoted by the amine itself, when this is basic enough (this being the reason why in many cases one works at molar ratios pyrylium: amine of 1:2 when pyridinium salts are prepared by this method), or by external catalysts added to the reaction mixture, such as triethylamine [38, 39]. The derivatives **56** are generally unstable, being tautomers with the ketodieneamines **57**, which are the key intermediates for

the conversion of pyryliums into pyridiniums [38, 45–47]. In acidic media, in the rate-determining step of the whole process, ketodieneamines **57** may be converted to the corresponding pyridinium salts **58**, although other products, such as vinylogous amides with diverse structures have also been isolated in such reactions [46, 47]. A supplementary complication appears when the moiety substituting the 2- and/or 6-position(s) of the pyrylium ring is methyl, case in which a concurrent cyclisation with formation of the anilines **59** in addition to the pyridinium salts **58**, may take place too [39, 46, 48]. These concurrent reactions mentioned above are generally important when the amine to be converted into the pyridinium salt possesses weak nucleophilicity or basicity. This happens to be the case of sulfanilamide **9a**. In fact, reaction of **9a** with several pyrylium salts, performed in a variety of conditions (different solvents, such as low molecular weight alcohols (MeOH, EtOH, *i*-PrOH); DMF; methylene chloride; acetonitrile; diverse molar ratios of the reagents; temperatures from 25 to 150 °C; reaction times between 15 min and 48 h, etc.) led only to the isolation of the unreacted raw materials. The only conditions which led to the formation of the pyridinium salts **11–22** were the following: anhydrous methanol in the presence of acetic anhydride as solvent and triethylamine as catalysts for the deprotonation of the intermediates **55**. Acetic anhydride had the role of reacting with the water formed in the condensation reaction. This water may in fact act as a competitive nucleophile with sulfanilamide when reacting with the pyrylium cation, and as a consequence the yields in pyridinium salts would dramatically be decreased [47]. After the rapid formation of the ketodieneamine, catalyzed by triethylamine (and in the presence of the acetic anhydride as water scavenging agent), the cyclisation to the pyridinium ring (the rate-determining step) has been achieved by prolonged refluxation in the presence of acetic acid (2–5 h, see Experimental protocols for details). Still the yields were not always good, especially for the 2-methyl-containing derivatives. Less complications were registered in the case of homosulfanilamide **9b** and even fewer for 4-(2-aminoethyl)-benzenesulfonamide **9c**, which was generally converted to the pyridinium derivatives with the best yields (see Experimental protocols for details).

A large variety of R^2–R^6 moieties substituting the pyrylium salts **10** have been chosen, since it was shown in previous works from this laboratory that the nature of these moieties is the determining factor for the biological activity as enzyme inhibitors of the obtained pyridinium salts [13, 29]. In a QSAR study [13] for thiadiazole-sulfonamides of type **7**, possessing the same type of moieties substituting the pyridinium ring as the new inhibitors reported by us here, it was demonstrated that the strongest CA II inhibitors were obtained when the moieties substituting the 2,6-positions of the pyridinium ring were aliphatic groups such as methyl, ethyl or/and *iso*-propyl, whereas the 4-position was occupied by an aromatic (generally phenyl) moiety. Much weaker inhibitors were obtained for 2,4,6-triaryl- or 2-alkyl-4,6-diarylpyridinium moieties substituting the thiadiazole-sulfonamide in the series of derivatives **7** [13]. Thus, in the present study a larger series of 2,6-dialkyl-4-phenylpyridinium salts of sulfanilamide, homosulfanilamide and 4-(2-aminoethyl)-benzenesulfonamide has been synthesized, but for the sake of a complete SAR insight for these series of CA inhibitors, the 2,4,6-triphenyl-, as well as 2-alkyl-4,6-diphenylpyridinium derivatives have also been obtained and assayed for inhibition of three CA isozymes, together with a relatively small number of 2,6-di- and 2,3,4,6-tetrasubstituted pyridinium derivatives (*table I*). The new compounds synthesized (three derivatives were reported previously, whereas 41 are new compounds) have been characterized by elemental analysis (± 0.4% of the theoretical data, calculated for the proposed formulas, for C, H, N and S) and spectroscopic methods (IR, electronic and NMR spectroscopy) which confirmed their structure (see Experimental protocols).

Sulfonamides **11–54** were assayed for inhibition of the following isozymes: hCA I, hCA II and bCA IV (h = human; b = bovine isozyme) (*table I*). The following observations should be remarked regarding the CA inhibitory properties of derivatives **11–54**: (i) pyridinium salts derived from 4-(2-aminoethyl)-benzenesulfonamide were more inhibitory than the corresponding homosulfanilamide derivatives, which in turn were more inhibitory than the corresponding sulfanilamide pyridinium salts, for all groups R^2–R^6 substituting the pyridinium moieties, and against all three CA isozymes. This observation might be correlated with one of our QSAR findings [13, 30, 49] which showed that the inhibitory power of a given compound increased with the length of its molecule in the direction of the axis passing through the Zn(II) ion of the enzyme, the nitrogen atom of the sulfonamido moiety coordinated to the metal ion and the heterocyclic ring of the inhibitor. This type of behavior has been observed both for heterocyclic sulfonamides as well as aromatic derivatives, bearing positively-charged [13], or Schiff base [49] moieties in their molecule, as well as for a non-congeneric series of inhibitors with the general formula RSO_2NH_2 [30]. Thus, the longer molecules of derivatives **39–54** (two carbon atoms separating the pyridinium and benzenesulfonamide rings) acted as more powerful inhibitors than the homosulfanilamides **23–38**

Table I. Inhibition of isozymes hCA I, hCA II and bCA IV with the pyridinium salts **11–54**.



Compound	n	R^2	R^3	R^4	R^6	K_I[a]		
						hCA I[b] (μM)	hCA II[b] ($\times 10^8$ M)	bCA IV[c] ($\times 10^8$ M)
11	0	Me	H	Me	Me	10 ± 0.5	15 ± 1.7	29 ± 1.0
12	0	Me	H	Ph	Me	7 ± 1.1	6 ± 0.8	21 ± 1.1
13	0	Et	H	Ph	Et	6 ± 0.5	6 ± 0.3	18 ± 0.2
14	0	*n*-Pr	H	Ph	*n*-Pr	10 ± 0.6	12 ± 1.1	19 ± 0.4
15	0	*i*-Pr	H	Ph	*i*-Pr	5 ± 0.4	5 ± 0.2	9 ± 0.6
16	0	Me	H	Ph	Ph	40 ± 0.4	21 ± 0.7	85 ± 2.2
17	0	Et	H	Ph	Ph	43 ± 0.2	40 ± 2.5	130 ± 0.9
18	0	*n*-Pr	H	Ph	Ph	140 ± 2.5	58 ± 1.1	148 ± 3.6
19	0	*i*-Pr	H	Ph	Ph	125 ± 0.7	44 ± 0.9	210 ± 2.4
20	0	*n*-Bu	H	Ph	Ph	305 ± 10	62 ± 1.0	215 ± 5.1
21	0	Ph	H	Ph	Ph	290 ± 5.2	51 ± 3.0	250 ± 9.0
22	0	Me	Me	Me	Me	5 ± 0.5	4 ± 0.2	6 ± 0.1
23	1	Me	H	Me	Me	7 ± 0.1	5 ± 0.2	9 ± 0.2
24	1	*i*-Pr	H	Me	Me	6 ± 0.1	5 ± 1.1	8 ± 0.9
25	1	*i*-Pr	H	Me	*i*-Pr	11 ± 0.5	8 ± 1.3	14 ± 0.4
26	1	Me	H	Ph	Me	4 ± 0.2	2 ± 0.3	7 ± 0.5
27	1	Et	H	Ph	Et	2 ± 0.9	2 ± 0.3	5 ± 0.2
28	1	*n*-Pr	H	Ph	*n*-Pr	24 ± 1.1	9 ± 0.2	16 ± 0.3
29	1	*i*-Pr	H	Ph	*i*-Pr	12 ± 0.1	6 ± 0.1	10 ± 0.5
30	1	Me	H	Ph	Ph	32 ± 0.2	12 ± 0.1	16 ± 1.0
31	1	Et	H	Ph	Ph	42 ± 1.4	31 ± 1.4	98 ± 3.2
32	1	*n*-Pr	H	Ph	Ph	130 ± 5.3	39 ± 1.7	126 ± 2.0
33	1	*i*-Pr	H	Ph	Ph	112 ± 2.1	37 ± 0.8	121 ± 1.4
34	1	*n*-Bu	H	Ph	Ph	300 ± 5.9	59 ± 3.5	210 ± 2.4
35	1	*t*-Bu	H	Ph	Ph	110 ± 3.6	32 ± 1.1	107 ± 0.4
36	1	Ph	H	Ph	Ph	280 ± 4.5	47 ± 2.9	195 ± 6.0
37	1	Ph	H	H	Ph	280 ± 0.1	49 ± 3.7	195 ± 2.4
38	1	Me	Me	Me	Me	3 ± 0.1	3 ± 0.5	5 ± 0.9
39	2	Me	H	Me	Me	4 ± 0.2	2 ± 0.1	6 ± 0.6
40	2	*i*-Pr	H	Me	Me	2 ± 0.3	1.5 ± 0.1	3 ± 0.2
41	2	*i*-Pr	H	Me	*i*-Pr	3 ± 0.1	2 ± 0.1	7 ± 0.1
42	2	Me	H	Ph	Me	1 ± 0.2	0.8 ± 0.2	2 ± 0.3
43	2	Et	H	Ph	Et	1 ± 0.5	0.9 ± 0.2	2 ± 0.1
44	2	*n*-Pr	H	Ph	*n*-Pr	7 ± 0.1	4 ± 0.2	8 ± 0.2
45	2	*i*-Pr	H	Ph	*i*-Pr	6 ± 0.1	2 ± 1.1	7 ± 0.5
46	2	Me	H	Ph	Ph	18 ± 0.5	10 ± 1.3	14 ± 0.4
47	2	Et	H	Ph	Ph	40 ± 0.2	22 ± 0.3	76 ± 3.1
48	2	*n*-Pr	H	Ph	Ph	112 ± 4.9	27 ± 0.3	105 ± 5.0
49	2	*i*-Pr	H	Ph	Ph	94 ± 3.1	35 ± 0.2	86 ± 3.9
50	2	*n*-Bu	H	Ph	Ph	290 ± 8.5	54 ± 4.0	200 ± 8.5
51	2	*t*-Bu	H	Ph	Ph	92 ± 2.8	27 ± 1.5	100 ± 6.0
52	2	Ph	H	Ph	Ph	270 ± 10.5	41 ± 1.9	183 ± 4.0
53	2	Ph	H	H	Ph	265 ± 2.0	42 ± 1.0	190 ± 5.1
54	2	Me	Me	Me	Me	2 ± 0.1	1 ± 0.1	2 ± 0.1

[a] Mean ± standard error ($n = 2$); [b] human (cloned) isozymes; [c] from bovine lung microsomes.

Table II. CA inhibition data with standard sulfonamide inhibitors **1–5**, **8** and **9a–c**.

Inhibitor	K_I[a]		
	hCA I[b] (μM)	hCA II[b] ($\times 10^8$ M)	bCA IV[c] ($\times 10^8$ M)
Acetazolamide **1**	0.90 ± 0.01	1.2 ± 0.05	22 ± 0.09
Benzolamide **2**	0.78 ± 0.02	1.4 ± 0.02	24 ± 0.10
Ethoxzolamide **3**	0.25 ± 0.01	0.81 ± 0.01	1.32 ± 0.04
Dichlorophenamide **4**	12 ± 0.05	3.80 ± 0.08	38 ± 0.12
Dorzolamide **5**	59 ± 0.15	0.9 ± 0.07	4.31 ± 0.13
QAS **8**	115 ± 4	90 ± 12	355 ± 15
Sulfanilamide **9a**	28 ± 0.14	30 ± 0.20	300 ± 1.56
Homosulfanilamide **9b**	25 ± 0.62	17 ± 0.18	280 ± 2.04
4-(2-Aminoethyl)benzenesulfonamide **9c**	21 ± 0.54	18 ± 0.25	245 ± 1.90

[a] Mean ± standard error ($n = 2$) ; [b] human (cloned) isozymes ; [c] from bovine lung microsomes.

(one sp^3 carbon atom between the two structural elements), which in turn were more inhibitory than the sulfanilamides **11–22**, which possessed the 'shortest' molecule. These data are valid for the same type of substitution patterns at the pyridinium ring, in the three series of investigated CA inhibitors; (ii) the most powerful inhibitory effects were observed for compounds possessing the following substitution at the pyridinium ring: only alkyl moieties (such as the 2,4,6-trimethyl-, 2,3,4,6-tetramethyl- or 2-*iso*-propyl-4,6-dimethyl-pyridinium) or such groups as 4-phenyl-2,6-dialkyl (2,6-dimethyl-4-phenyl- and 2,6-diethyl-4-phenyl-pyridinium), in all three series of sulfonamides prepared by us here. As for derivatives **7** [13, 29, 30], substitution patterns including two or three aryl (phenyl) groups linked to the pyridinium ring led to much weaker inhibitors, probably due to steric hindrance effects and impaired access to the active site of the enzyme; (iii) the susceptibility of the three isozymes to inhibition with this class of inhibitors was: hCA II > bCA IV >> hCA I, similarly to classical inhibitors, such as acetazolamide **1**, benzolamide **2**, dichlorophenamide **4** or sulfanilamide **9a** (*table II*) [4, 10]. Thus, up to now the only compounds showing an enhanced affinity for CA IV (of the same order of magnitude with that for CA II, or slightly better) are the Schiff bases of type **6** reported by this group [11, 12, 16]. Still, some of the new derivatives reported here, such as **23**, **24**, **27**, **38**, **40**, **42–44**, **46** and **54**, showed selectivity ratios (K_I(CA IV)/K_I(CA II)) in the range of 1.4-2.5, whereas the same ratio for classical inhibitors is generally in the range of 5–13, proving that a slight progress of enzyme selectivity has been achieved. Of course this is not enough for claiming a selective inhibition (in vitro), but due to the special physicochemical properties of the new inhibitors, this effect was achieved in vivo (see later in the text). One should also mention that some of the most active derivatives in the prepared series (such as **26**, **27**, **40–43** and **54**) act as quite powerful inhibitors, with potencies comparable to those of the classical sulfonamides **1–5**.

The above differences of affinity for the different isozymes of the new inhibitors reported in this paper, and the fact that the X-ray crystallographic structure of hCA IV has been recently reported [50] and became available in the Brookhaven Protein Database (in September 1997; file code 1ZNC), prompted us to compare the tridimensional structure and active site architecture for the three CA isozymes (CA I, II and IV) used in the present work (human and bovine CA IV possess very similar kinetic and inhibition susceptibility properties [51]) in order to explain their affinity for the sulfonamide inhibitors reported by us here.

Thus, *figures 5–7* show some active site residues known to be important for catalysis in isozymes I, II and IV [33, 50, 52, 53] (the files 1HUH for hCA I and 2CBA for hCA II have been used, also available from Brookhaven Protein Database [52, 53]).

It has previously been proposed by us [33] that the high catalytic efficiency of the most active isozyme, hCA II, is due to a unique feature of its active site: the presence of a histidine cluster, consisting of the residues: His 64 – two conformations; His 4 – two conformations, His 3, His 10, His 15, His 17 (*figure 5*) [33]. This cluster extends from the interior of the active site (His 64) to its entrance (His 4 and His 3) till the surface of the protein (in the proximity of the active site entrance) and it probably constitutes a very appropriate 'channel' for efficiently transferring protons from the active site to the reaction medium, but also for the binding of amphipatic compounds, such as the sulfonamide inhibitors [33, 54]. As seen from *figure 6*, in the low activity isozyme hCA I

(which also binds sulfonamide inhibitors with a 10-100 times lower affinity), such a cluster does not exist [33]. Moreover, the pathways for the proton transfer are somehow bifurcated and divergent as the four histidines present within the active site, i.e., His 64, 67, 200 and 243 (excepting, of course, for the three Zn(II) ligands, which in all isozymes are His 94, 96 and 119) are placed at bifurcating positions. As seen from *figure 6*, these four histidines in hCA I (His 64, His 67, His 200 and His 243) are rather buried in the active site so that, probably, the proton transfer cannot be as efficient as the one assisted by the histidine cluster present in hCA II, whereas inhibitors are probably unable to interact with them, as documented by crystallographic studies of adducts of hCA I with sulfonamides and anion inhibitors [52]. In this context it is also enlightening that histamine, the first activator of hCA II for which the X-ray crystal structure has recently been reported by this group [33], which is able to enhance the catalytic efficiency of the enzyme, does really bind in the region of the active site of hCA II containing the histidine cluster shown in *figure 5*. In this context, we suggest a new hypothesis for explaining also differences of affinity of the discussed CA isozymes for the sulfonamide inhibitors, based on the interaction of inhibitors with the structural elements of the active site mentioned above. Indeed, for example in hCA II the amino acid residue in position 4 is His, whereas in hCA I it is an Asp. This residue, situated just at the entrance within the active site, is a constituent of the histidine cluster of isozyme II. The negative charge of the carboxylate group of the Asp residue in hCA I probably influences its interaction with positively-charged inhibitors of the type described by us here. In fact many such compounds (such as **22**, **27**, **38–43** or **54**) have a higher affinity for hCA I as compared to uncharged sulfonamides of the classical type (**9a–c**), presumably due to a supplementary interaction between this negative charge and the pyridinium moiety of the inhibitor molecule.

In the case of hCA IV (*figure 7*) only one histidine residue is present within the active site, His 64, which as in hCA II, plays a critical role in catalysis, as proton shuttle residue between the active site and the environment. But the most characteristic feature of the active site of this isozyme is related to the presence of four cysteine residues, which form two disulfide bonds, situated at the entrance within the active site cavity (Cys 6-Cys 11G, and Cys 23-Cys 203, respectively). These residues occupy practically the same region of the active site as the histidine cluster in hCA II, and we consider this as the most relevant aspect explaining the difference in affinity for sulfonamide inhibitors of the two isozymes. The hypothesis of Stams et al. [50] that it is the residue 131 (a valine in hCA IV and a phenylalanine in hCA II) which is responsible for the above mentioned differences, seems to us very unrealistic, since this residue is situated relatively far away from the active site, and could account for such a difference in affinity only for inhibitors with an extremely long and flexible molecule.

Although the inhibitors designed by us here are not isozyme-specific for hCA IV, the isozyme selectivity has been achieved in vivo and ex vivo, due to the presence of the positive charges in their molecules, which, as a consequence became membrane-impermeable (*table III*). From data of *table III* it can be seen that incubation of human red cells (which contain very high concentrations of isozymes I and II, i.e., 150 μM hCA I and 20 μM hCA II, but not the membrane-bound CA IV [55]) with millimolar concentrations of different sulfonamide inhibitors, led to saturation of the two isozymes with inhibitor in the case of acetazolamide, sulfanilamide and dorzolamide, already after short periods of incubation (30 min), whereas for benzolamide a similar effect is achieved after somehow longer periods (60 min) (*table III*). This is obviously due to the high diffusibility through membranes of the first three inhibitors, whereas benzolamide with a pK_a of 3.2 for the second sulfonamido group [56] being present mainly as an (di)anion at the pH at which the experiment has been done (7.4), is already less diffusible and penetrates membranes in a longer time. Different cationic sulfonamides synthesized by us here, such as **27**, **40**, **42**, **43**, **54**, in the same conditions, were detected only in very small amounts within the blood red cells, proving that they were unable to penetrate through membranes, obviously due to their cationic nature. Even after incubation times as long as one hour, only traces of such cationic sulfonamides were present inside the blood red cells, as proved by the three assay methods used for their identification in the cell lysate, which were in good agreement with each other (see Experimental protocols for details).

In order to prove that in vivo, membrane-bound CA, but not cytosolic isozymes are inhibited by the cationic sulfonamides reported by us here, additional experiments of i.v. perfusion in rats have been performed and the amount of bicarbonate excreted into urine has been analyzed, together with the level of sulfonamides present in red cells (*table IV*) [6, 57–60]. It is well established [6, 57–61] that at least two CA isozymes, CA II and IV, are involved in bicarbonate reabsorption by the proximal and to a less extent also distal tubules in the kidneys of vertebrates (except those of *Reptilia*, in which other mechanisms were shown to be present [61]). Administration of CA inhibitors (such as acetazolamide, benzolamide, ethoxzolamide, etc.) to vertebrates produces an

Figure 5. Detail of the hCA II active site with catalytic residues and other groups thought to be important for inhibitor binding. The histidine cluster, extending from within the middle of the active site till the surface of the enzyme is particularly evidenced.

alkaline urine, due to the renal excretion of bicarbonate, Na^+, K^+ and osmotically obligated water, as a consequence of CA inhibition [61]. Up to now, studies of selective inhibition of different CA isozymes present in the diverse compartments of renal tubular cells have been performed only with high molecular weight CA inhibitors

Figure 6. Detail of the hCA I active site with catalytic residues and other groups thought to be important for inhibitor binding.

of types **60–62** (see *figure 8*) [6, 58–60], with molecular weights in the range of 3.5–99 kDa, which being membrane-impermeant due to their high molecular weight, were shown to selectively inhibit only CA IV and not the cytosolic isozymes (primarily CA II) [6, 58–60]. The particular nature of the compounds reported by us

Figure 7. Detail of the hCA IV active site with catalytic residues and other groups thought to be important for inhibitor binding.

here allows for the first time to perform this type of study with low molecular weight inhibitors.

From data of *table IV* one can see that administration of classical low molecular weight inhibitors, such as acetazolamide **1** or benzolamide **2** (which inhibit both cytosolic as well as membrane-bound isozymes) leads to a peak of 105–110 mM of bicarbonate excreted into urine in 12 h, as shown in the classical studies of Maren's

Table III. Levels of sulfonamides (μM) in red blood cells at 30 and 60 min, after exposure of 10 mL of blood to solutions of sulfonamide inhibitor (2 mM sulfonamide in 5 mM Tris buffer, pH 7.4). The concentrations of sulfonamide have been determined by three methods: HPLC, electronic spectroscopy (ES) and the enzymatic method (EI) (see Experimental protocols for details).

Inhibitor	[Sulfonamide], μM[a]					
	t = 30 min			t = 60 min		
	HPLC[b]	ES[c]	EI[d]	HPLC[b]	ES[c]	EI[d]
Acetazolamide **1**	136 ± 7	139 ± 5	140 ± 4	160 ± 8	167 ± 5	163 ± 5
Benzolamide **2**	110 ± 5	108 ± 3	112 ± 2	148 ± 4	146 ± 6	149 ± 2
Sulfanilamide **9a**	148 ± 4	151 ± 5	143 ± 3	159 ± 3	164 ± 5	159 ± 5
Dorzolamide **5**	156 ± 6	158 ± 9	159 ± 5	163 ± 1	168 ± 4	164 ± 5
27	0.9 ± 0.1	0.5 ± 0.03	0.6 ± 0.01	0.8 ± 0.01	0.5 ± 0.01	0.5 ± 0.03
40	1.2 ± 0.1	1.2 ± 0.3	1.0 ± 0.1	1.1 ± 0.2	1.3 ± 0.2	1.1 ± 0.1
42	0.8 ± 0.07	1.0 ± 0.09	0.9 ± 0.1	0.9 ± 0.05	1.2 ± 0.1	1.0 ± 0.07
43	1.0 ± 0.2	0.6 ± 0.04	0.8 ± 0.1	0.9 ± 0.06	1.1 ± 0.09	1.2 ± 0.2
54	0.8 ± 0.07	0.7 ± 0.05	0.7 ± 0.04	1.1 ± 0.2	1.3 ± 0.1	0.9 ± 0.1

[a] Mean ± standard deviation (from 2 determinations) by: [b] the HPLC method; [c] the electronic spectroscopic method; [d] the enzymatic method.

group [61], and reconfirmed in the present work. Administration of positively-charged sulfonamides such as **40** and **54**, in the same conditions as for the classical inhibitors mentioned above, produced bicarbonate elimination, but in a lower quantity, due to the fact that only CA IV was inhibited by these membrane-impermeant compounds. On the other hand, after this experiments, only traces of positively-charged sulfonamides have been detected into the red cells of the experimental animals, in contrast with the situation after the administration of acetazolamide or benzolamide, which produced saturation of the cytosolic isozymes. Our data also compare well with the recent data from Maren's laboratory [6] who reported an excretion of about 40 mM bicarbonate into urine, also in the rat, after 100 mg/kg of the 3.5 kDa inhibitor **60**. In their work it was concluded that both CA II and CA IV participate in the normal full renal reabsorption of bicarbonate [6], these data being in agreement with data of Sly's group [62] and obviously with the data reported by us here. Thus, corroborating the results of *tables III* and *IV*, it is clear that ex vivo and in vivo, positively-charged sulfonamides as those described here are able to discriminate between membrane-bound and cytosolic isozymes. This is not surprizing after all,

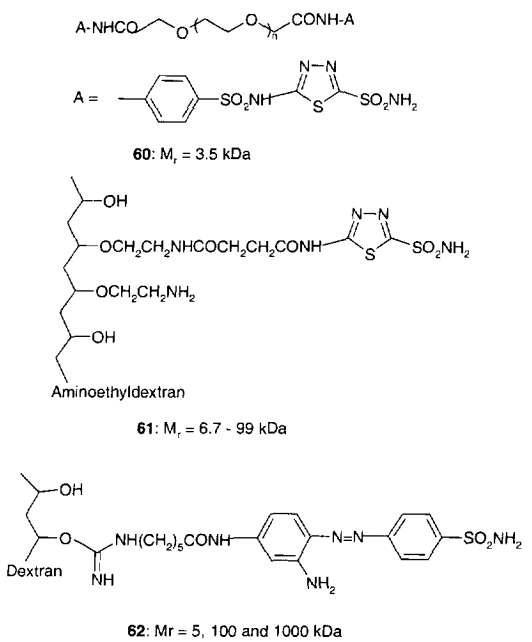


Figure 8.

Table IV. Renal excretion of bicarbonate in rats, after administration of the classical sulfonamide CA inhibitors acetazolamide **1** and benzolamide **2**, as well as the positively charged sulfonamides **40** and **54**, and the level of sulfonamide present in red cells 2 h after administration of the drug.

Inhibitor	Drug concentration [mg/kg]	Excreted HCO_3^- [mM]	[Sulfonamide][a] [μM]
Acetazolamide	10	110 ± 3	164 ± 9
Benzolamide	5	115 ± 6	155 ± 8
40	5	42 ± 3	0.02 ± 0.009
54	3	44 ± 2	0.01 ± 0.002

[a] By the HPLC method.

since many positively-charged compounds, such as some reversible anticholinesterase agents (neostigmine; edrophonium; pyridostigmine; demecarium; etc.) [63]; the quaternary ammonium antimuscarinic agents (such as methantheline) [64]; or the neuromuscular blocking agents (tubocurarine; alcuronium; gallamine; atracurium; decamethonium; etc.) [65], all possessing quaternary ammonium or pyridinium moieties in their molecules, are known to be membrane-impermeant due to their cationic nature.

5. Conclusions

A large series of cationic aromatic sulfonamides has been prepared by reaction of sulfanilamide, homosulfanilamide or 4-(2-aminoethyl)-benzenesulfonamide with di-, tri- or tetrasubstituted pyrylium salts bearing alkyl, aryl or combination of the two moieties in their molecule. Qualitative SAR proved that best activity for inhibiting isozymes I, II and IV of CA was obtained for compounds bearing a 4-aryl- and 2,6-dialkyl groups substituting the pyridinium moiety of the new compounds, with the 4-(2-aminoethyl)-benzenesulfonamide derivatives more active than the corresponding homosulfanilamides, which in turn were more active than the corresponding sulfanilamides. Although the obtained inhibitors do not prove isozyme specificity in vitro, selective inhibition of the membrane-bound isozyme CA IV has been demonstrated in vivo, even in the presence of high amounts of the other (cytosolic) isozymes. This is due to the membrane-impermeability of this new class of inhibitors. Thus, this is the first study in which such an effect has been achieved with low molecular weight CA inhibitors, since up to now only polymeric such compounds were able to selectively inhibit CA IV. Our results open new vistas for obtaining more selective drugs from the class of CA inhibitors, or for physiological studies in which membrane-impermeable derivatives are essential. A hypothesis is presented to explain the difference in affinity for sulfonamide inhibitors of the major, physiologically relevant isozymes, CA I, II and IV, based on the number of histidine residues present within their active site cavity.

6. Experimental protocols

6.1. Chemistry

Melting points were recorded with a heating plate microscope and are not corrected. IR spectra were recorded in KBr pellets with a Carl Zeiss UR-20 instrument, whereas UV spectra in methanol as solvent, with a Specord C. Zeiss double beam or Cary 3 spectrophotometers. ^{1}H-NMR spectra were recorded in trifluoroacetic acid (TFA) as solvent with a Varian-60A or Bruker CPX200 instruments. Chemical shifts are reported as (values, relative to Me_4Si as standard. Elemental analysis was done by combustion (for C, H, N) with a Carlo Erba automated analyzer (Milan, Italy). The values obtained were within ± 0.4% of the theoretical values, calculated for the proposed formulae. HPLC was performed with a Beckman instrument, using a Rheodyne pump and column (reverse phase, 5 μm Bondapack C18).

Sulfanilamide **9a**, homosulfanilamide hydrochloride **9b**, 4-(2-aminoethyl)-benzenesulfonamide **9c** and the other sulfonamides used as standards in the enzymatic assay were from Aldrich, Merck and Carlo Erba. Benzolamide was a gift from dr. T.H. Maren (University of Florida, Gainesville). Pyrylium salts were prepared by literature procedures, generally by olefin (or their precursors) bisacylation, as described in the literature [38, 66–68].

Human CA I and CA II cDNAs were expressed in *Escherichia coli* strain BL21 (DE3) from the plasmids pACA/hCA I and pACA/hCA II described by Forsman et al. [69] (the two plasmids were a gift from dr. Sven Lindskog, Umea University, Sweden). Cell growth conditions were those described by Lindskog's group [70], and enzymes were purified by affinity chromatography according to the method of Khalifah et al. [71]. 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 hCA I, and 29.3 kDa for hCA II, respectively [72, 73]. bCA IV was isolated from bovine lung microsomes as described by Maren et al., and its concentration has been determined by titration with ethoxzolamide [31].

*6.1.1. General procedure for the preparation of compounds **11–22***

An amount of 2.9 mM of sulfanilamide **9a** and 2.9 mM of pyrylium salt **10** were suspended in 5 mL of anhydrous methanol and poured into a stirred mixture of 14.5 mM of triethylamine and 5.8 mM of acetic anhydride. After five minutes of stirring, another 10 mL of methanol were added to the reaction mixture, which was heated to reflux for 15 min. Then 14.5 mM of acetic acid was added and heating was continued for 2–5 h. The role of the acetic anhydride is to react with the water formed during the condensation reaction between the pyrylium salt and the aromatic amine, in order to shift the equilibrium towards the formation of the pyridinium salts of type **11–22**. In the case of sulfanilamide, this procedure is the only one which gave acceptable yields in pyridinium salts, probably due to the deactivating effect of the sulfamoyl moiety on the amine group, which becomes poorly nucleophilic and unreactive towards these reagents. The precipitated pyridinium salts obtained were purified by treatment with concentrated ammonia solution (which also converts the eventually unreacted pyrylium salt to the corresponding pyridine which is soluble in acidic medium), reprecipitation with perchloric acid and recrystallization from water with 2–5% $HClO_4$.

*6.1.2. General procedure for the preparation of compounds **23–38***

*Preparation of homosulfanilamide **9b** free base*: The commercially available homosulfanilamide hydrochloride was dissolved into the minimum amount of water and brought to pH 8.5 with a 6 N NaOH solution. The precipitated free base was filtered,

thoroughly washed with distilled water and recrystallized from water (m.p. 150–151 °C, no m.p. of the free base is specified in [74]).

Preparation of the pyridinium salts: An amount of 0.5 g (2.68 mM) of free base **9b** prepared as described above and the stoichiometric amount of pyrylium salt **10** and triethylamine (2.68 mM) were dissolved in 20 mL of absolute methanol. The mixture was refluxed for 30 min, then 5.4 mM of glacial acetic acid were added and refluxation was continued for other 2 h. The cold mixture was treated with 100-200 mL of diethyl ether for the precipitation of the pyridinium salts which were recrystallized from water with 2-5% perchloric acid.

*6.1.3. General procedure for the preparation of compounds **39–54***

An amount of 2.5 mM of 4-(2-aminoethyl)-benzenesulfonamide **9c** and 2.5 mM of pyrylium perchlorate **10** were dissolved in 15 mL of methanol, and 2.5 mM of triethylamine were added. The mixture was refluxed for 15 min, when an amount of 5 mM of glacial acetic acid was added and refluxation continued for other 45–60 min. The reaction mixture was left overnight at 0 °C, the eventual precipitate obtained was then collected by filtration and purified as described above for the sulfanilamide derivatives **11–22**. When no precipitation occurred, an excess of diethyl ether (100–250 mL) was added to the reaction mixture, and the obtained oily precipitate purified similarly to the precipitate mentioned above.

*1-N-(4-Sulfamoylphenyl)-2,4,6-trimethylpyridinium perchlorate **11***, white crystals, m.p. 255–258 °C (yield of 12%); IR (KBr), cm^{-1}: 625, 740, 1100, 1170, 1342, 1630, 3040, 3245, 3335; UV (MeOH), λ_{max}, nm (lg ε): 220 sh (4.54), 275 (3.89); ^{1}H-NMR (TFA), δ, ppm: 2.51 (s, 3H, 4-Me); 2.72 (s, 6H, 2,6-$(Me)_2$); 7.35-7.90 (m, AA′BB′, 4H, ArH from 1,4-phenylene); 8.10 (s, 2H, ArH, 3,5-H from pyridinium). Anal. $C_{14}H_{17}N_2O_2S^+$ ClO_4^- (C, H, N, S).

*1-N-(4-Sulfamoylphenyl)-2,6-dimethyl-4-phenylpyridiniumperchlorate **12***, white crystals, m.p. 286–288 °C (yield of 55%); IR (KBr), cm^{-1}: 625, 690, 770, 1100, 1170, 1330, 1635, 3030, 3260, 3330; λ_{max}, nm (lg : ε): 218 sh (4.37), 294 (4.42); ^{1}H-NMR (TFA), δ, ppm: 2.62 (s, 6H, 2,6-$(Me)_2$); 8.10-9.12 (m, 11H, ArH from 1,4-phenylene, pyridinium and 4-Ph). Anal. $C_{19}H_{19}N_2O_2S^+$ ClO_4^- (C, H, N, S).

*1-N-(4-Sulfamoylphenyl)-2,6-diethyl-4-phenylpyridiniumchlorate **13***, yellow crystals, m.p. 216–218 °C (yield of 24%); IR (KBr), cm^{-1}: 625, 695, 765, 1100, 1180, 1340, 1630, 3040, 3270, 3360; UV (MeOH), λ_{max}, nm (lg ε): 219 sh (4.34), 294 (4.38); ^{1}H-NMR (TFA), δ, ppm: 1.43 (t, 6H, 2 Me from ethyl); 2.82 (q, 4H, 2 CH_2 from Et); 7.68-8.87 (m, 11H, ArH from 1,4-phenylene, pyridinium and 4-Ph). Anal. $C_{21}H_{23}N_2O_2S^+$ ClO_4^- (C, H, N, S).

*1-N-(4-Sulfamoylphenyl)-2,6-di-n-propyl-4-phenylpyridinium perchlorate **14***, yellowish crystals, m.p. 180–181 °C (yield of 61%); IR (KBr), cm^{-1}: 625, 695, 770, 1100, 1180, 1340, 1630, 3050, 3220, 3315; UV (MeOH), λ_{max}, nm (lg ε): 220 sh (4.40), 295 (4.44); ^{1}H-NMR (TFA), δ, ppm: 1.01 (t, 6H, 2 Me from propyl); 1.70 (sextet, 4H, 2CH_2 (β) from *n*-Pr); 2.80 (t, 4H, 2 CH_2 (α) from *n*-Pr); 7.55-8.78 (m, 11H, ArH from 1,4-phenylene, pyridinium and 4-Ph). Anal. $C_{23}H_{27}N_2O_2S^+$ ClO_4^- (C, H, N, S).

*1-N-(4-Sulfamoylphenyl)-2,6-di-isopropyl-4-phenylpyridinium perchlorate **15***, white crystals, m.p. 257–259 °C (yield of 15%); IR (KBr), cm^{-1}: 625, 690, 765, 1100, 1180, 1340, 1625, 3040, 3270, 3315; UV (MeOH), λ_{max}, nm (lg ε): 220 sh (4.38), 293 (4.42); ^{1}H-NMR (TFA), δ, ppm: 1.45 (d, 12H, 4 Me from *i*-Pr); 2.95 (heptet, 2H, 2 CH from *i*-Pr); 7.92-8.97 (m, 11H, ArH from 1,4-phenylene, pyridinium and 4-Ph). Anal. $C_{23}H_{27}N_2O_2S^+$ ClO_4^- (C, H, N, S).

*1-N-(4-Sulfamoylphenyl)-2-methyl-4,6-diphenylpyridinium perchlorate **16***, white crystals, m.p. 128–130 °C (yield of 7%); IR (KBr), cm^{-1}: 625, 710, 770, 1100, 1170, 1345, 1625, 3040, 3245, 3350; UV (MeOH), λ_{max}, nm (lg ε): 222 (4.71), 307 (4.58); ^{1}H-NMR (TFA), δ, ppm: 2.72 (s, 3H, 2-Me); 7.55-8.73 (m, 16H, ArH from 1,4-phenylene, pyridinium and 4,6-Ph_2). Anal. $C_{24}H_{21}N_2O_2S^+$ ClO_4^- (C, H, N, S).

*1-N-(4-Sulfamoylphenyl)-2-ethyl-4,6-diphenylpyridinium perchlorate **17***, white-yellow crystals, m.p. 133–135 °C (yield of 36%); IR (KBr), cm^{-1}: 625, 700, 770, 1100, 1180, 1340, 1620, 3040, 3250, 3350; UV (MeOH), λ_{max}, nm (lg ε): 220 sh (4.57), 306 (4.52); ^{1}H-NMR (TFA), δ, ppm: 1.50 (t, 3H, Me from ethyl); 2.97 (q, 2H, CH_2); 7.40-8.57 (m, 16H, ArH from 1,4-phenylene, pyridinium and 4,6-Ph_2). Anal. $C_{25}H_{23}N_2O_2S^+$ ClO_4^- (C, H, N, S).

*1-N-(4-Sulfamoylphenyl)-2-n-propyl-4,6-diphenylpyridinium perchlorate **18***, white crystals, m.p. 204–205 °C (yield of 33%); IR (KBr), cm^{-1}: 625, 700, 770, 1100, 1180, 1340, 1620, 3030, 3270, 3350; UV (MeOH), λ_{max}, nm (lg ε): 221 (4.82), 305 (4.51); ^{1}H-NMR (TFA), δ, ppm: 1.05 (t, 3H, Me from propyl); 1.93 (sextet, 2H, (-CH_2 from *n*-Pr); 2.93 (t, 2H, (-CH_2 from *n*-Pr); 7.38-8.53 (m, 16H, ArH from 1,4-phenylene, pyridinium and 4,6-Ph_2). Anal. C_{26}H25N2O2S$^+$ ClO_4^- (C, H, N, S).

*1-N-(4-Sulfamoylphenyl)-2-iso-propyl-4,6-diphenylpyridinium perchlorate **19***, white crystals, m.p. 138–140 °C (yield of 12%); IR (KBr), cm^{-1}: 625, 700, 770, 1100, 1170, 1340, 1620, 3040, 3250, 3360; UV (MeOH), λ_{max}, nm (lg (): 221.5 (4.67), 305 (4.35); ^{1}H-NMR (TFA), (, ppm: 1.52 (d, 6H, 2 Me from *i*-propyl); 2.52-3.25 (m, 1H, CH from *i*-Pr); 7.33-8.60 (m, 16H, ArH from 1,4-phenylene, pyridinium and 4,6-Ph_2). Anal. $C_{26}H_{25}N_2O_2S^+$ ClO_4^- (C, H, N, S).

*1-N-(4-Sulfamoylphenyl)-2-n-butyl-4,6-diphenylpyridinium perchlorate **20***, white crystals, m.p. 248–250 °C (yield of 70%); IR (KBr), cm^{-1}: 625, 710, 770, 1100, 1180, 1335, 1625, 3040, 3260, 3345; UV (MeOH), λ_{max}, nm (lg ε): 221 (4.80), 304 (4.59); ^{1}H-NMR (TFA), δ, ppm: 0.90 (t, 3H, Me from butyl); 1.10-2.15 (m, 4H, CH_3–CH_2–CH_2–CH_2 from *n*-Bu); 2.97 (t, 2H, (-CH_2 from *n*-Bu); 7.25-8.52 (m, 16H, ArH from 1,4-phenylene, pyridinium and 4,6-Ph_2). Anal. $C_{27}H_{27}N_2O_2S^+$ ClO_4^- (C, H, N, S).

*1-N-(4-Sulfamoylphenyl)-2,4,6-triphenylpyridinium perchlorate **21***, yellow crystals, m.p. 162–165 °C (yield of 80%); IR (KBr), cm^{-1}: 625, 700, 770, 1100, 1170, 1340, 1620, 3030, 3260, 3350; UV (MeOH), λ_{max}, nm (lg ε): 236 sh (4.31), 315 (4.57); ^{1}H-NMR (TFA), δ, ppm: 7.47-8.63 (m, 21H, ArH from 1,4-phenylene, pyridinium and 2,4,6-Ph_3). Anal. $C_{29}H_{23}N_2O_2S^+$ ClO_4^- (C, H, N, S).

*1-N-(4-Sulfamoylphenyl)-2,3,4,6-tetramethylpyridinium perchlorate **22***, white crystals, m.p. 250–251 °C (yield of 22%); IR

(KBr), cm^{-1}: 625, 750, 1100, 1170, 1340, 1630, 3040, 3245, 3330; UV (MeOH), λ_{max}, nm (lg ε): 221 sh (4.49), 275 (3.87); ^{1}H-NMR (TFA), δ, ppm: 2.45 (s, 3H, 3-Me); 2.50 (s, 3H, 4-Me); 2.55 (s, 3H, 6-Me); 2.75 (s, 3H, 2-Me); 8.03-9.17 (m, 5H, ArH from 1,4-phenylene and pyridinium 5-H). Anal. $C_{15}H_{19}N_2O_2S^+ ClO_4^-$ (C, H, N, S).

1-N-(4-Sulfamoylphenylmethyl)-2,4,6-trimethylpyridinium perchlorate ***23***, white-tan crystals, m.p. 168–170 °C, lit. [39] m.p. 166-8 °C; (yield of 67%); IR (KBr), cm^{-1}: 625, 680, 1100, 1170, 1330, 1635, 3030, 3250; UV (MeOH), λ_{max}, nm (lg ε): 230 (4.20), 271 (3.84); ^{1}H-NMR (TFA), δ, ppm: 2.72 (s, 3H, 4-Me); 2.85 (s, 6H, 2,6-$(Me)_2$); 6.06 (s, 2H, CH_2); 7.13-8.41 (m, AA'BB', 4H, ArH from 1,4-phenylene); 8.00 (s, 2H, ArH, 3,5-H from pyridinium). Anal. $C_{15}H_{19}N_2O_2S^+ ClO_4^-$ (C, H, N, S).

1-N-(4-Sulfamoylphenylmethyl)-2-iso-propyl-4,6-dimethylpyridinium perchlorate ***24***, light orange crystals, m.p. 192–194 °C; (yield of 50%); IR (KBr), cm^{-1}: 625, 680, 720, 1100, 1165, 1330, 1640, 3020, 3235; UV (MeOH), λ_{max}, nm (lg : 227 (4.32), 273 (3.98); ^{1}H-NMR (TFA), δ, ppm: 1.50 (d, 6H, 2Me from *i*-Pr); 2.80 (s, 3H, 6-Me); 2.90 (s, 3H, 4-Me); 3.48 (heptet, 1H, CH from *i*-Pr); 6.15 (s, 2H, CH_2); 7.25-8.43 (m, AA'BB', 4H, ArH from 1,4-phenylene); 7.98 (s, 2H, ArH, 3,5-H from pyridinium). Anal. $C_{17}H23N2O2S^+ ClO_4^-$ (C, H, N, S).

1-N- (4-Sulfamoylphenylmethyl)-2,6-di-iso-propyl-4-methylpyridinium perchlorate ***25***, tan crystals, m.p. 171–173 °C; (yield of 50%); IR (KBr), cm^{-1}: 625, 685, 820, 1100, 1165, 1340, 1635, 3030, 3250; UV (MeOH), λ_{max}, nm (lg ε): 227 (4.26), 274 (3.94); ^{1}H-NMR (TFA), δ, ppm: 1.51 (d, 12H, 4Me from 2 *i*-Pr); 2.83 (s, 3H, 4-Me); 3.42 (heptet, 2H, 2CH from 2 *i*-Pr); 6.25 (s, 2H, CH_2); 7.31-8.51 (m, AA'BB', 4H, ArH from 1,4-phenylene); 8.03 (s, 2H, ArH, 3,5-H from pyridinium). Anal. $C_{19}H_{27}N_2O_2S^+ ClO_4^-$ (C, H, N, S).

1-N-(4-Sulfamoylphenylmethyl)-2,6-dimethyl-4-phenylpyridinium perchlorate ***26***, orange-red crystals, m.p. 198–200 °C, (yield of 66%); IR (KBr), cm^{-1}: 625, 765, 1100, 1165, 1345, 1630, 3050, 3265; UV (MeOH), λ_{max}, nm (lg ε): 223.5 sh (4.28), 294 (4.32); ^{1}H-NMR (TFA), δ, ppm: 3.00 (s, 6H, 2,6-$(Me)_2$); 6.20 (s, 2H, CH_2); 7.21-8.51 (m, 11H, ArH from 1,4-phenylene, 4-Ph and 3,5-H from pyridinium). Anal. $C_{20}H_{21}N_2O_2S^+ ClO_4^-$ (C, H, N, S).

1-N-(4-Sulfamoylphenylmethyl)-2,6-diethyl-4-phenylpyridinium perchlorate ***27***, tan crystals, m.p. 203–206 °C, (yield of 53%); IR (KBr), cm^{-1}: 625, 770, 1100, 1160, 1330, 1630, 3060, 3230; UV (MeOH), λ_{max}, nm (lg ε): 223.5 sh (4.32), 292 (4.20); ^{1}H-NMR (TFA), δ, ppm: 1.55 (t, 6H, 2 Me from Et); 3.30 (q, 4H, 2 CH_2 from Et); 6.23 (s, 2H, N^+–CH_2); 7.08-8.63 (m, 11H, ArH from 1,4-phenylene, 4-Ph and 3,5-H from pyridinium). Anal. $C_{22}H_{25}N_2O_2S^+ ClO_4^-$ (C, H, N, S).

1-N-(4-Sulfamoylphenylmethyl)-2,6-di-n-propyl-4-phenylpyridinium perchlorate ***28***, tan crystals, m.p. 208–210 °C, (yield of 50%); IR (KBr), cm^{-1}: 625, 775, 1100, 1160, 1320, 1630, 3060, 3240; UV (MeOH), λ_{max}, nm (lg ε): 229 (4.30), 294 (4.40); ^{1}H-NMR (TFA), δ, ppm: 1.15 (t, 6H, 2 Me from Pr); 1.90 (sextet, 4H, 2 CH_2 from Pr); 3.18 (t, 4H, 2 CH_2 from Pr); 6.20 (s, 2H, N^+–CH_2); 7.10-8.50 (m, 11H, ArH from 1,4-phenylene, 4-Ph and 3,5-H from pyridinium). Anal. $C_{24}H_{29}N_2O_2S^+ ClO_4^-$ (C, H, N, S).

1-N-(4-Sulfamoylphenylmethyl)-2,6-di-iso-propyl-4-phenylpyridinium perchlorate ***29***, tan crystals, m.p. 211–213 °C, (yield of 44%); IR (KBr), cm^{-1}: 625, 775, 1100, 1165, 1320, 1625, 3060, 3240; UV (MeOH), λ_{max}, nm (lg ε): 227 (4.27), 291 (4.24); ^{1}H-NMR (TFA), δ, ppm: 1.55 (d, 12H, 4 Me from *i*-Pr); 3.53 (heptet, 2H, 2 CH from *i*-Pr); 6.32 (s, 2H, N^+–CH_2); 7.23-8.65 (m, 11H, ArH from 1,4-phenylene, 4-Ph and 3,5-H from pyridinium). Anal. $C_{24}H_{29}N_2O_2S^+ ClO_4^-$ (C, H, N, S).

1-N-(4-Sulfamoylphenylmethyl)-2-methyl-4,6-diphenylpyridinium perchlorate ***30***, yellow crystals, m.p. 241–243 °C, lit. [39] m.p. 241–3 °C (yield of 58%); IR (KBr), cm^{-1}: 625, 770, 1100, 1170, 1320, 1625, 3050, 3250; UV (MeOH), λ_{max}, nm (lg ε): 225 sh (4.36), 303 (4.40); ^{1}H-NMR (TFA), δ, ppm: 3.00 (s, 3H, 2-Me); 6.07 (s, 2H, CH_2); 7.08-8.58 (m, 16H, ArH from 1,4-phenylene, 4,6-Ph_2 and 3,5-H from pyridinium). Anal. $C_{25}H_{23}N_2O_2S^+ ClO_4^-$ (C, H, N, S).

1-N-(4-Sulfamoylphenylmethyl)-2-ethyl-4,6-diphenylpyridinium perchlorate ***31***, white crystals, m.p. 123–125 °C, (yield of 52%); IR (KBr), cm^{-1}: 625, 705, 770, 1100, 1160, 1335, 1620, 3050, 3250; UV (MeOH), λ_{max}, nm (lg ε): 223.5 (4.50), 303 (4.43); ^{1}H-NMR (TFA), δ, ppm: 1.60 (t, 3H, Me from Et); 3.27 (q, 2H, CH_2 from Et); 6.05 (s, 2H, N^+–CH_2); 7.08-8.60 (m, 16H, ArH from 1,4-phenylene, 4,6-Ph_2 and 3,5-H from pyridinium). Anal. $C_{26}H_{25}N_2O_2S^+ ClO_4^-$ (C, H, N, S).

1-N-(4-Sulfamoylphenylmethyl)-2-n-propyl-4,6-diphenylpyridinium perchlorate ***32***, white-yellowish crystals, m.p. 132–133.5 °C, (yield of 57%); IR (KBr), cm^{-1}: 625, 685, 770, 1100, 1165, 1345, 1620, 3080, 3250; UV (MeOH), λ_{max}, nm (lg ε): 225 (4.40), 303 (4.45); ^{1}H-NMR (TFA), δ, ppm: 1.18 (t, 3H, Me from Pr); 2.10 (sextet, 2H, CH_2 from *n*-Pr); 3.20 (t, 2H, CH_2 from *n*-Pr); 6.05 (s, 2H, N^+–CH_2); 7.08-8.63 (m, 16H, ArH from 1,4-phenylene, 4,6-Ph_2 and 3,5-H from pyridinium). Anal. $C_{27}H_{27}N_2O_2S^+ ClO_4^-$ (C, H, N, S).

1-N-(4-Sulfamoylphenylmethyl)-2-iso-propyl-4,6-diphenylpyridinium perchlorate ***33***, tan crystals, m.p. 128–129 °C, (yield of 53%); IR (KBr), cm^{-1}: 625, 710, 770, 1100, 1160, 1335, 1620, 3070, 3250; UV (MeOH), λ_{max}, nm (lg ε): 227 (4.44), 303 (4.46); ^{1}H-NMR (TFA), δ, ppm: 1.55 (d, 6H, 2 Me from *i*-Pr); 3.55 (heptet, 1H, CH from *i*-Pr); 6.10 (s, 2H, N^+–CH_2); 7.08-8.63 (m, 16H, ArH from 1,4-phenylene, 4,6-Ph_2 and 3,5-H from pyridinium). Anal. $C_{27}H_{27}N_2O_2S^+ ClO_4$ (C, H, N, S).

1-N-(4-Sulfamoylphenylmethyl)-2-n-butyl-4,6-diphenylpyridinium perchlorate ***34***, tan crystals, m.p. 168–169 °C, (yield of 52%); IR (KBr), cm^{-1}: 625, 690, 770, 1100, 1160, 1340, 1620, 3080, 3250; UV (MeOH), λ_{max}, nm (lg ε): 225 (4.40), 303 (4.43); ^{1}H-NMR (TFA), δ, ppm: 0.93 (t, 3H, Me from *n*-Bu); 1.55 (sextet, 2H, CH_2 from *n*-Bu); 2.05 (quintet, 2H, CH_2 from *n*-Bu); 3.17 (t, 2H, CH_2 from *n*-Bu); 6.05 (s, 2H, $N^+ - CH_2$); 7.08-8.58 (m, 16H, ArH from 1,4-phenylene, 4,6-Ph_2 and 3,5-H from pyridinium). Anal. $C_{28}H_{29}N_2O_2S^+ ClO_4^-$ (C, H, N, S).

1-N-(4-Sulfamoylphenylmethyl)-2-tert-butyl-4,6-diphenylpyridinium perchlorate ***35***, white crystals, m.p. 179–180 °C (yield of 42%); IR (KBr), cm^{-1}: 625, 705, 765, 1100, 1160, 1310, 1620, 3060, 3270; UV (MeOH), λ_{max}, nm (lg ε): 227 (4.10), 308 (4.20); ^{1}H-NMR (TFA), δ, ppm: 1.90 (s, 9H, *t*-Bu); 6.45 (s, 2H, CH_2);

6.83-8.83 (m, 16H, ArH from 1,4-phenylene, 4,6-Ph_2 and 3,5-H from pyridinium). Anal. $C_{28}H_{29}N_2O_2S^+ ClO_4^-$ (C, H, N, S).

1-N-(4-Sulfamoylphenylmethyl)-2,4,6-triphenylpyridinium tetrafluoroborate ***36***, orange crystals, m.p. 135–137 °C, lit. [39] m.p. 134–136 °C (yield of 73%); IR (KBr), cm^{-1}: 610, 705, 770, 1090, 1165, 1335, 1620, 3050, 3270; UV (MeOH), λ_{max}, nm (lg ε): 224 sh (4.46), 311 (4.43); ^{1}H-NMR (TFA), δ, ppm: 6.06 (s, 2H, CH_2); 6.70-8.56 (m, 21H, ArH from 1,4-phenylene, 2,4,6-Ph_3 and 3,5-H from pyridinium). Anal. $C_{30}H_{25}N_2O_2S^+ BF_4^-$ (C, H, N, S).

1-N-(4-Sulfamoylphenylmethyl)-2,6-diphenylpyridinium perchlorate ***37***, yellow-orange crystals, m.p. 118–120 °C (yield of 20%); IR (KBr), cm^{-1}: 625, 705, 765, 1100, 1160, 1335, 1615, 3050, 3260; UV (MeOH), λ_{max}, nm (lg ε): 216 (4.42), 221 (4.55), 235 sh (4.16), 308 (4.13), 500 (3.19); ^{1}H-NMR (TFA), δ, ppm: 6.13 (s, 2H, CH_2); 6.71-8.40 (m, 17H, ArH from 1,4-phenylene, 2,6-Ph_2 and 3,4,5-H from pyridinium). Anal. $C_{24}H_{21}N_2O_2S^+ ClO_4^-$ (C, H, N, S).

1-N-(4-Sulfamoylphenylmethyl)-2,3,4,6-tetramethylpyridinium perchlorate ***38***, white-tan crystals, m.p. 253–255 °C; (yield of 65%); IR (KBr), cm^{-1}: 625, 800, 1100, 1165, 1330, 1630, 3030, 3305; UV (MeOH), λ_{max}, nm (lg ε): 225 (4.25), 278 (3.90); ^{1}H-NMR (TFA), δ, ppm: 2.60 (s, 3H, 4-Me); 2.77 (s, 3H, 3-Me); 2.87 (s, 6H, 2,6-$(Me)_2$); 6.15 (s, 2H, CH_2); 7.21-8.50 (m, AA'BB', 4H, ArH from 1,4-phenylene); 7.90 (s, 1H, ArH, 5-H from pyridinium). Anal. $C_{16}H_{21}N_2O_2S^+ ClO_4^-$ (C, H, N, S).

1-N-(4-Sulfamoylphenylethyl)-2,4,6-trimethylpyridinium perchlorate ***39***, white crystals, m.p. 155–157 °C (yield of 53%); IR (KBr), cm^{-1}: 625, 680, 1100, 1165, 1330, 1635, 3060, 3250, 3330; UV (MeOH), λ_{max}, nm (lg ε): 227 (4.01), 273 (3.61); ^{1}H-NMR (TFA), δ, ppm: 2.66 (s, 3H, 4-Me); 2.88 (s, 6H, 2,6-$(Me)_2$); 3.42 (t, 2H, CH_2); 4.92 (t, 2H, CH_2); 7.47-8.38 (m, 6H, ArH from 1,4-phenylene and 3,5-H from pyridinium). Anal. $C_{16}H_{21}N_2O_2S^+ ClO_4^-$ (C, H, N, S).

1-N-(4-Sulfamoylphenylethyl)-2-iso-propyl-4,6-dimethylpyridinium perchlorate ***40***, white crystals, m.p. 185–187 °C; (yield of 43%); IR (KBr), cm^{-1}: 625, 685, 1100, 1170, 1340, 1635, 3040, 3255, 3380; UV (MeOH), λ_{max}, nm (lg ε): 224.5 (4.08), 272 (3.78); ^{1}H-NMR (TFA), δ, ppm: 1.47 (d, 6H, 2Me from *i*-Pr); 2.68 (s, 3H, 4-Me); 2.90 (s, 3H, 6-Me); 3.13-3.70 (m, 3H, CH from *i*-Pr + CH_2); 5.00 (t, 2H, CH_2); 7.33-8.35 (m, 6H, ArH from 1,4-phenylene and 3,5-H from pyridinium). Anal. $C_{18}H_{25}N_2O_2S^+ ClO_4^-$ (C, H, N, S).

1-N-(4-Sulfamoylphenylethyl)-2,6-di-iso-propyl-4-methylpyridinium perchlorate ***41***, white crystals, m.p. 256–257 °C; (yield of 53%); IR (KBr), cm^{-1}: 625, 685, 1100, 1170, 1340, 1630, 3040, 3235, 3410; UV (MeOH), λ_{max}, nm (lg ε): 225 (4.23), 273 (3.98); ^{1}H-NMR (TFA), δ, ppm: 1.48 (d, 12H, 4Me from 2 *i*-Pr); 2.70 (s, 3H, 4-Me); 3.15-3.77 (m, 4H, 2CH from 2 *i*-Pr + CH_2); 5.02 (t, 2H, CH_2); 7.33-8.27 (m, 6H, ArH from 1,4-phenylene and 3,5-H from pyridinium). Anal. $C_{20}H_{29}N_2O_2S^+ ClO_4^-$ (C, H, N, S).

1-N-(4-Sulfamoylphenylethyl)-2,6-dimethyl-4-phenylpyridinium perchlorate ***42***, white crystals, m.p. 201–203 °C, (yield of 65%); IR (KBr), cm^{-1}: 625, 690, 780, 1100, 1165, 1330, 1630, 3050, 3280; UV (MeOH), λ_{max}, nm (lg ε): 223 (4.18), 292 (4.27); ^{1}H-NMR (TFA), δ, ppm: 3.08 (s, 6H, 2,6-$(Me)_2$); 3.50 (t, 2H, CH_2); 5.00 (t, 2H, CH_2); 7.55-8.37 (m, 11H, ArH from 1,4-phenylene, 4-Ph and 3,5-H from pyridinium). Anal. $C_{21}H_{23}N_2O_2S^+ ClO_4^-$ (C, H, N, S).

1-N-(4-Sulfamoylphenylethyl)-2,6-diethyl-4-phenylpyridinium perchlorate ***43***, white crystals, m.p. 221–223 °C, (yield of 58%); IR (KBr), cm^{-1}: 625, 700, 780, 1100, 1165, 1335, 1630, 3060, 3240, 3335; UV (MeOH), λ_{max}, nm (lg ε): 219 (4.32), 294 (4.39); ^{1}H-NMR (TFA), δ, ppm: 1.67 (t, 6H, 2 Me from Et); 3.15-3.80 (m, 6H, 2 CH_2 from Et + CH_2 from ethylene bridge); 5.07 (t, 2H, CH_2 from ethylene bridge); 7.57-8.50 (m, 11H, ArH from 1,4-phenylene, 4-Ph and 3,5-H from pyridinium). Anal. $C_{23}H_{27}N_2O_2S^+ ClO_4^-$ (C, H, N, S).

1-N-(4-Sulfamoylphenylethyl)-2,6-di-n-propyl-4-phenylpyridinium perchlorate ***44***, white crystals, m.p. 218–219 °C, (yield of 66%); IR (KBr), cm^{-1}: 625, 685, 775, 1100, 1170, 1330, 1630, 3050, 3255, 3335; UV (MeOH), λ_{max}, nm (lg ε): 219 (4.43), 294 (4.44); ^{1}H-NMR (TFA), δ, ppm: 1.23 (t, 6H, 2 Me from Pr); 2.03 (q, 4H, 2 CH_2 from Pr); 3.07-3.75 (m, 6H, 2 CH_2 from Pr + CH_2 from ethylene bridge); 5.03 (t, 2H, CH_2 from ethylene bridge); 7.55-8.43 (m, 11H, ArH from 1,4-phenylene, 4-Ph and 3,5-H from pyridinium). Anal. $C_{25}H_{31}N_2O_2S^+ ClO_4^-$ (C, H, N, S).

1-N-(4-Sulfamoylphenylethyl)-2,6-di-iso-propyl-4-phenylpyridinium perchlorate ***45***, white crystals, m.p. 246–248 °C, (yield of 66%); IR (KBr), cm^{-1}: 625, 685, 765, 1100, 1170, 1325, 1630, 3060, 3270, 3350; UV (MeOH), λ_{max}, nm (lg ε): 219 (4.40), 293 (4.42); ^{1}H-NMR (TFA), δ, ppm: 1.60 (d, 12H, 4 Me from *i*-Pr); 3.32-3.83 (m, 4H, 2 CH from *i*-Pr + CH_2 from ethylene bridge); 5.13 (t, 2H, CH_2 from ethylene bridge); 7.47-8.43 (m, 11H, ArH from 1,4-phenylene, 4-Ph and 3,5-H from pyridinium). Anal. $C_{25}H_{31}N_2O_2S^+ ClO_4^-$ (C, H, N, S).

1-N-(4-Sulfamoylphenylethyl)-2-methyl-4,6-diphenylpyridinium perchlorate ***46***, white crystals, m.p. 239–241 °C (yield of 35%); IR (KBr), cm^{-1}: 625, 675, 775, 1100, 1165, 1340, 1625, 3050, 3245, 3435; UV (MeOH), λ_{max}, nm (lg ε): 223 (4.30), 301 (4.33); ^{1}H-NMR (TFA), δ, ppm: 3.03-3.37 (m, 5H, 2-Me + CH_2 from ethylene bridge); 4.93 (t, 2H, CH_2 from ethylene bridge); 7.05-8.45 (m, 16H, ArH from 1,4-phenylene, 4,6-Ph_2 and 3,5-H from pyridinium). Anal. $C_{26}H_{25}N_2O_2S^+ ClO_4^-$ (C, H, N, S).

1-N-(4-Sulfamoylphenylethyl)-2-ethyl-4,6-diphenylpyridinium perchlorate ***47***, white crystals, m.p. 209–210 °C, (yield of 58%); IR (KBr), cm^{-1}: 625, 685, 750, 1100, 1170, 1340, 1620, 3050, 3220, 3390; UV (MeOH), λ_{max}, nm (lg ε): 223 (4.44), 302 (4.45); ^{1}H-NMR (TFA), δ, ppm: 1.72 (t, 3H, Me from Et); 2.92-3.78 (m, 4H, CH_2 from Et + CH_2 from ethylene bridge); 4.93 (t, 2H, CH_2 from ethylene bridge); 6.88-8.47 (m, 16H, ArH from 1,4-phenylene, 4,6-Ph_2 and 3,5-H from pyridinium). Anal. $C_{27}H_{27}N_2O_2S^+ ClO_4^-$ (C, H, N, S).

1-N-(4-Sulfamoylphenylethyl)-2-n-propyl-4,6-diphenylpyridinium perchlorate ***48***, white crystals, m.p. 218–219 °C, (yield of 53%); IR (KBr), cm^{-1}: 625, 705, 775, 1100, 1170, 1340, 1620, 3080, 3255, 3340; UV (MeOH), λ_{max}, nm (lg ε): 224 (4.42), 301 (4.44); ^{1}H-NMR (TFA), δ, ppm: 1.32 (t, 3H, Me from Pr); 2.17 (sextet, 2H, CH_2 from *n*-Pr); 2.82-3.63 (m, 4H, CH_2 from *n*-Pr + CH_2 from ethylene bridge); 4.98 (t, 2H, CH_2 from ethylene

bridge); 6.83-8.43 (m, 16H, ArH from 1,4-phenylene, 4,6-Ph_2 and 3,5-H from pyridinium). Anal. $C_{28}H_{29}N_2O_2S^+ ClO_4^-$ (C, H, N, S).

1-N-(4-Sulfamoylphenylethyl)-2-iso-propyl-4,6-diphenylpyridinium perchlorate ***49***, white crystals, m.p. 133–135 °C, (yield of 40%); IR (KBr), cm^{-1}: 625, 700, 765, 1100, 1165, 1340, 1620, 3070, 3250, 3350; UV (MeOH), λ_{max}, nm (lg ε): 223.5 (4.44), 302 (4.44); ^{1}H-NMR (TFA), δ, ppm: 1.70 (d, 6H, 2 Me from *i*-Pr); 3.25 (t, 2H, CH_2 from ethylenic bridge); 3.50-4.03 (m, 1H, CH from *i*-Pr); 4.95 (t, 2H, CH_2 from ethylene bridge); 6.95-8.53 (m, 16H, ArH from 1,4-phenylene, 4,6-Ph_2 and 3,5-H from pyridinium). Anal. $C_{28}H_{29}N_2O_2S^+ ClO_4^-$ (C, H, N, S).

1-N-(4-Sulfamoylphenylethyl)-2-n-butyl-4,6-diphenylpyridinium perchlorate ***50***, white crystals, m.p. 196–198 °C, (yield of 79%); IR (KBr), cm^{-1}: 625, 685, 7650, 1100, 1170, 1335, 1620, 3080, 3255, 3330; UV (MeOH), λ_{max}, nm (lg ε): 223 (4.43), 303 (4.45); ^{1}H-NMR (TFA), δ, ppm: 1.15 (t, 3H, Me from *n*-Bu); 1.38-2.45 (m, 4H, 2 CH_2 from *n*-Bu); 3.00-3.68 (m, 4H, CH_2 from *n*-Bu + CH_2 from ethylene bridge); 4.97 (t, 2H, CH_2 from ethylene bridge); 7.02-8.43 (m, 16H, ArH from 1,4-phenylene, 4,6-Ph_2 and 3,5-H from pyridinium). Anal. $C_{29}H_{31}N_2O_2S^+ ClO_4^-$ (C, H, N, S).

1-N-(4-Sulfamoylphenylethyl)-2-tert-butyl-4,6-diphenylpyridinium perchlorate ***51***, white crystals, m.p. 135–136 °C (yield of 42%); IR (KBr), cm^{-1}: 625, 700, 765, 1100, 1165, 1335, 1620, 3060, 3250, 3370; UV (MeOH), λ_{max}, nm (lg ε): 225 (4.42), 309.5 (4.44); ^{1}H-NMR (TFA), δ, ppm: 1.92 (s, 9H, *t*-Bu); 3.20 (t, 2H, CH_2); 5.33 (t, 2H, CH_2 from ethylene bridge); 6.90-8.77 (m, 16H, ArH from 1,4-phenylene, 4,6-Ph_2 and 3,5-H from pyridinium). Anal. $C_{29}H_{31}N_2O_2S^+ ClO_4^-$ (C, H, N, S).

1-N-(4-Sulfamoylphenylethyl)-2,4,6-triphenylpyridinium perchlorate ***52***, yellow crystals, m.p. 212–214 °C (yield of 73%); IR (KBr), cm^{-1}: 625, 680, 770, 1100, 1170, 1340, 1615, 3050, 3260, 3335; UV (MeOH), λ_{max}, nm (lg ε): 223.5 (4.50), 310 (4.48); ^{1}H-NMR (TFA), δ, ppm: 3.03 (t, 2H, CH_2 from ethylene bridge); 4.85 (t, 2H, CH_2 from ethylene bridge); 6.57-8.40 (m, 21H, ArH from 1,4-phenylene, 2,4,6-Ph_3 and 3,5-H from pyridinium). Anal. $C_{31}H_{26}N_2O_2S^+ ClO_4^-$ (C, H, N, S).

1-N-(4-Sulfamoylphenylethyl)-2,6-diphenylpyridinium perchlorate ***53***, yellow crystals, m.p. 205–206 °C (yield of 11%); IR (KBr), cm^{-1}: 625, 700, 760, 1100, 1165, 1350, 1615, 3050, 3240, 3325; UV (MeOH), λ_{max}, nm (lg ε): 223 (4.35), 300 (3.94), 500 (3.22); ^{1}H-NMR (TFA), δ, ppm: 2.98 (t, 2H, CH_2); 4.97 (t, 2H, CH_2 from ethylene bridge); 6.55-8.50 (m, 17H, ArH from 1,4-phenylene, 2,6-Ph_2 and 3,4,5-H from pyridinium). Anal. $C_{25}H_{23}N_2O_2S^+ ClO_4^-$ (C, H, N, S).

1-N-(4-Sulfamoylphenylethyl)-2,3,4,6-tetramethylpyridinium perchlorate ***54***, white crystals, m.p. 179–181 °C; (yield of 55%); IR (KBr), cm^{-1}: 625, 680, 1100, 1165, 1340, 1620, 3030, 3245, 3325; UV (MeOH), λ_{max}, nm (lg ε): 225.5 (4.26), 278 (3.88); ^{1}H-NMR (TFA), δ, ppm: 2.52 (s, 3H, 3-Me); 2.62 (s, 3H, 4-Me); 2.83 (s, 3H, 6-Me); 2.92 (s, 3H, 2-Me); 3.38 (t, 2H, CH_2); 4.93 (t, 2H, CH_2); 7.61-8.55 (m, 5H, ArH from 1,4-phenylene + 5-H from pyridinium). Anal. $C_{16}H_{21}N_2O_2S^+ ClO_4^-$ (C, H, N, S).

6.2. *Pharmacology*

6.2.1. *Carbonic anhydrase inhibition*

Initial rates of 4-nitrophenyl acetate hydrolysis catalyzed by different CA isozymes were monitored spectrophotometrically, at 400 nm, with a Cary 3 instrument interfaced with an IBM compatible PC [75]. Solutions of substrate 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, in the conditions of the experiments (pH 7.40), as reported in the literature [75]. Non-enzymic hydrolysis rates were always subtracted from the observed rates. Duplicate experiments were done for each inhibitor concentration, and the values reported throughout the paper are the mean of such results. Stock solutions of inhibitor (1 mM) were prepared in distilled-deionized water with 10–20% (v/v) DMSO (which is not inhibitory at these concentrations [21, 22]) and dilutions up to 0.01 nM were done thereafter with distilled-deionized water. Inhibitor and enzyme solutions were preincubated together for 10 min at room temperature prior to assay, in order to allow for the formation of the E-I complex. The inhibition constant K_I was determined as described by Pocker and Stone [75]. Enzyme concentrations were 3.5 nM for CA II, 10 nM for CA I and 36 nM for CA IV (this isozyme has a decreased esterase activity [32] and higher concentrations had to be used for the measurements). CA activity in homogenates of red cells was determined similarly.

6.2.2. *Penetrability through red cell mebranes*

An amount of 10 mL of freshly isolated human red cells thoroughly washed several times with Tris buffer (pH 7.40, 5 mM) and centrifuged for 10 min were treated with 25 mL of a 2 mM solution of sulfonamide inhibitor. Incubation has been done at 37 °C with gentle stirring, for periods of 30–60 min. After that time, the red cells were centrifuged again for 10 min, the supernatant discarded, and the cells washed three times with 10 mL of the above mentioned buffer, in order to eliminate all unbound inhibitor. The cells were then lysed in 25 mL of distilled water, centrifuged for eliminating membranes and other insoluble impurities. The obtained solution was heated at 100 °C for 5 minutes and sulfonamides possibly present have been assayed in each sample by three methods: a HPLC method [76, 77], spectrophotometrically [78] and enzymatically [75].

HPLC: A variant of the methods of Gomaa [76] and Bonazzi et al. [77] has been developed by us, as follows: a commercially available 5 μm Bondapak C-18 column was used for the separation, with a mobile phase made of acetonitrile-methanol-phosphate buffer (pH 7.4) 10:2:88 (v/v/v), at a flow rate of 3 mL/min, with 0.3 mg/mL sulphadiazine (Sigma) as internal standard. The retention times were: 12.69 min for acetazolamide; 4.55 min for sulphadiazine; 10.54 min for benzolamide; 4.12 min for sulfanilamide; 6.25 min for dorzolamide; 2.13 min for **27**; 2.43 min for **40**; 2.40 min for **43**; 2.35 min for **43** and 1.90 min for **54**. The eluent was monitored continuously for absorbance (at 254 nm for acetazolamide, and wavelength in the range of 270–310 nm in the case of the other sulfonamides, generally the absorption maximum reported at the descritpion of the particular compounds, see below).

Spectrophotometrically: A variant of the pH-induced spectrophotometric assay of Abdine et al. [78] has been used, working for instance at 260 and 292 nm, respectively, for acetazolamide; at 225 and 265 nm, respectively, for sulfanilamide, etc. Standardized solutions of each inhibitor have been prepared in the same buffer as the one used for the membrane penetrability experiments.

Enzymatically: the amount of sulfonamide present in the lysate has been evaluated based on hCA II inhibition measured with the

esterase method, as described above [75]. Standard inhibition curves have been obtained previously for each sulfonamide, using the pure compound, which were used thereafter for determining the amount of inhibitor present in the lysate.

Mention should be made that the three methods presented above led to results in good agreement, within the limits of the experimental errors.

6.2.3. Intravenous perfusion experiments in rats

Adult male Wistar rats were perfused intravenously with solutions of sulfonamide CA inhibitors in the concentration ranges of 3–15 mg/kg (equal volumes of inhibitor solutions have been used in all experiments). Urine has been collected for the next 12 h and the amount of bicarbonate present in it has been determined enzymatically with a phosphoenol pyruvate carboxylase-malate dehydrogenase assay, with a kit from Gilford Systems (Oberlin, OH, USA) [79]. Three animals were used for each inhibitor, and the data reported in *table IV* are the mean of such assays. Small amounts (0.5 mL) of blood were also prelevated from these animals, at 1 and 2 h after starting the perfusion experiment in order to determine the level of sulfonamides present in the erythrocytes. Red cells were treated as above (except that they were not incubated with any inhibitor) and sulfonamides present in the cell lysate were measured by the HPLC method mentioned above.

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References

[1] Preceding part of this series: Supuran C.T. et al., Eur J. Med. Chem 33 (1998) 247–254.

[2] Hewett-Emmett D., Tashian R.E., Mol. Phylogenet. Evol. 5 (1996) 50–77.

[3] Henry R.P., Ann. Rev. Physiol. 58 (1996) 523–538.

[4] Supuran C.T., in: Puscas I. (Ed.), Carbonic Anhydrase and Modulation of Physiologic and Pathologic Processes in the Organism, Helicon, Timisoara, 1994, pp. 29–111.

[5] Winkler C.A., Kittelberger A.M., Schwartz G.J., Am. J. Physiol. 272 (1997) F551–560.

[6] Maren T.H., Conroy C.W., Wynns G.C., Godman D.R., J. Pharmacol. Exp. Ther. 280 (1997) 98–104.

[7] Dodgson S.J., Cherian K., Arch. Biochem. Biophys. 282 (1990) 1–7.

[8] Dodgson S.J., J. Appl. Physiol. 63 (1987) 2134–2141.

[9] Hazen S.A., Waheed A., Sly W.S., LaNoue K.F., Lynch C.J., FASEB J. 10 (1996) 481–490.

[10] Supuran C.T., Conroy C.W., Maren T.H., Eur. J. Med. Chem. 31 (1996) 843–846.

[11] Supuran C.T., Nicolae A., Popescu A., Eur. J. Med. Chem. 31 (1996) 431–438.

[12] Supuran C.T., Popescu A., Ilisiu M., Costandache A., Banciu M.D., Eur. J. Med. Chem. 31 (1996) 439–447.

[13] Supuran C.T., Clare B.W., Eur. J. Med. Chem. 30 (1995) 687–696.

[14] Supuran C.T., Scozzafava A., J. Enzyme Inhib. 12 (1997) 37–51.

[15] Briganti F., Pierattelli R., Scozzafava A., Supuran C.T., Eur. J. Med. Chem. 31 (1996) 1001–1010.

[16] Supuran C.T., Scozzafava A., Popescu A., Bobes-Tureac R., Banciu A., Creanga A., Bobes-Tureac G., Banciu M.D., Eur. J. Med. Chem. 32 (1997) 445–452.

[17] Bertini I., Briganti F., Scozzafava A., in: Berthon G. (Ed.), Handbook of Metal-Ligand Interactions of Biological Fluids (Vol. 1), Marcel Dekker, New York, 1995, pp. 175–191.

[18] Bertini I., Canti G., Luchinat C., Scozzafava A., J. Am. Chem. Soc. 100 (1978) 4873–4877.

[19] Bertini I., Luchinat C., Scozzafava A., Struct. Bonding 48 (1982) 45–92.

[20] Tu C., Paranawithana S.R., Jewell D.A., Tanhauser S.M., LoGrasso P.V., Wynns G.C., Laipis P.J., Silverman D.N., Biochemistry 29 (1990) 6400–6405.

[21] Puscas I., Supuran C.T., in: Coelho J. (Ed.), Aparelho Digestivo, MEDSI, Rio de Janeiro, 1996, pp. 1704–1734.

[22] Maren T.H., in: Botré F., Gros G., Storey B.T. (Eds.), Carbonic Anhydrase - From Biochemistry and Genetics to Physiology and Clinical Medicine, VCH, Weinheim, 1991, pp. 186–207.

[23] Bayer A., Ferrari F., Maren T.H., Erb C., J. Fr. Ophtalmol. 19 (1996) 357–362.

[24] Sugrue M.F., J. Ocul. Pharmacol. Ther. 12 (1996) 363–376.

[25] Weiner I.M., in: Gilman A.G., Rall T.W., Nies A.S., Taylor P. (Eds.), The Pharmacological Basis of Therapeutics (8th ed.), Pergamon Press, New York, 1990, pp. 713–732.

[26] Reiss W.G., Oles K.S., Ann. Pharmacother. 30 (1996) 514–519.

[27] Stoll M., Hamann G.F., Jost V., Bompotti U.A., Fitridge R., Schimrigk K., J. Neuroimag. 6 (1996) 144–149.

[28] Levine R.L., Tursky P.A., Turnipseed W.D., Grist T., J. Neuroimag. 6 (1996) 126–130.

[29] Supuran C.T. et al., J. Pharm. Sci. 81 (1992) 716–719.

[30] Clare B.W., Supuran C.T., Eur. J. Med. Chem. 32 (1997) 311–319.

[31] Maren T.H., Wynns G.C., Wistrand P.J., Mol. Pharmacol. 44 (1993) 901–906.

[32] Baird T.T., Waheed A., Okuyama T., Sly W.S., Fierke C.A., Biochemistry 36 (1997) 2669–2678.

[33] Briganti F., Mangani S., Orioli P., Scozzafava A., Vernaglione G., Supuran C.T., Biochemistry 36 (1997) 10384–10392.

[34] Supuran C.T., Balaban A.T., Gheorghiu M.D., Schiketanz A., Dinculescu A., Puscas I., Rev. Roum. Chim. 35 (1990) 399–405.

[35] Henry R.P., Am. J. Physiol. 252 (1987) R959–965.

[36] Bayer A., Ber. Deutsch. Chem. Ges. 43 (1910) 2337–2349.

[37] Bayer A., Piccard T., Liebigs Ann. Chem. 384 (1911) 208–223.

[38] Balaban A.T., Dinculescu A., Dorofeenko G.N., Fischer G.W., Koblik A.V., Mezheritskii V.V., Schroth W., in: Katritzky A.R. (Ed.), Advances in Heterocyclic Chemistry, Academic Press, New York, 1982, pp. 8–360.

[39] Dinculescu A., Balaban A.T., Rev. Roum. Chim. 25 (1980) 1505–1528.

[40] Supuran C.T., Pop E., Dinculescu A., Heterocycles 37 (1994) 667–671.

[41] Supuran C.T., Banciu M.D., Balaban A.T., Rev. Roum. Chim. 38 (1993) 199–205.

[42] Barboiu M., Supuran C.T., Luca C. et al., Liebigs Ann. (1996) 959–963.

[43] Barboiu M., Supuran C.T., Scozzafava A et al., Liebigs Ann. Rec. (1997) 1853–1859.

[44] Neidlein R., Witerzens F., Monats. Chem. 106 (1975) 643–648.

[45] Balaban A.T., Toma C., Tetrahedron Suppl. 7 (1966) 1–7.

[46] Katritzky A.R., Manzo R.H., J. Chem. Soc. Perkin Trans. 2 (1981) 571–575.

[47] Katritzky A.R., Lloyd J.M., Patel R.C., J. Chem. Soc. Perkin Trans. (1982) 117–123.

[48] Uncuta C., Balaban T.S., Petride A., Chiraleu F., Balaban A.T., Rev. Roum. Chim. 34 (1989) 1425–1437.

[49] Supuran C.T., Clare B.W., Eur. J. Med. Chem. 33 (1998) 489–500.

[50] Stams T. et al., Proc. Natl. Acad. Sci. USA 93 (1996) 13589–13594.

[51] Tamai S., Waheed A., Cody L.B., Sly W.S., Proc. Natl. Acad. Sci. USA 93 (1996) 13647–13652.

[52] Chakravarti S., Kannan K.K., J. Mol. Biol. 243 (1994) 298–309.

[53] Håkansson K., Carlsson M., Svensson L.A., Liljas A., J. Mol. Biol. 227 (1992) 1192–1204.

[54] Elbaum D., Nair S.K., Patchan M.W., Thompson R.B., Christianson D.W., J. Am. Chem. Soc. 118 (1996) 8381–8387.

[55] Wistrand P.J., Lindqvist A., in: Botré F., Gros G., Storey B.T. (Eds.), Carbonic Anhydrase – From Biochemistry and Genetics to Physiology and Clinical Medicine, VCH, Weinheim, 1991, pp. 352–374.

[56] Maren T.H., in: Karch F.E. (Ed.), Orphan Drugs, Marcel Dekker, New York, 1982, pp. 89–115.

[57] Wistrand P.J., Knuttila K.G., Kidney Int. 35 (1989) 851–859.

[58] Tinker J.P., Coulson R., Weiner I.M., J. Pharmacol. Exp. Ther. 218 (1981) 600–607.

[59] Lucci M.S., Tinker J.P., Weiner I.M., DuBose T.D., Am. J. Physiol. 245 (1983) F443–449.

[60] Heming T.A., Geers C., Gros G., Bidani A., Crandall E.D., J. Appl. Physiol. 61 (1986) 1849–1856.

[61] Maren T.H., Physiol. Rev. 47 (1967) 595–782.

[62] Sly W.S., Zhu X.L., Sato S., in: Botré F., Gros G., Storey B.T. (Eds.), Carbonic Anhydrase – From Biochemistry and Genetics to Physiology and Clinical Medicine, VCH, Weinheim, 1991, pp. 226–231.

[63] Taylor P., in: Gilman A.G., Rall T.W., Nies A.S., Taylor P. (Eds.), The Pharmacological Basis of Therapeutics (8th ed.), Pergamon Press, New York, 1990, pp. 131–149.

[64] Brown J.H., in: idem, pp. 150–166.

[65] Taylor P., in: idem, pp. 167–186.

[66] Balaban A.T., Nenitzescu C.D., Liebigs Ann. 625 (1959) 74–88.

[67] Supuran C.T., Baciu I., Balaban A.T., Rev. Roum. Chim. 38 (1993) 725–732.

[68] Supuran C.T., Barboiu M., Luca C., Pop E., Brewster M.E., Dinculescu A., Eur. J. Med. Chem. 31 (1996) 597–606.

[69] Forsman C., Behravan G., Osterman A., Jonsson B.H., Acta Chem. Scand. B42 (1988) 314–318.

[70] Behravan G., Jonasson P., Jonsson B.H., Lindskog S., Eur. J. Biochem. 198 (1991) 589–592.

[71] Khalifah R.G., Strader D.J., Bryant S.H., Gibson S.M., Biochemistry 16 (1977) 2241–2247.

[72] Nyman P.O., Lindskog S., Biochim. Biophys. Acta 85 (1964) 141–151.

[73] Henderson L.E., Henriksson D., Nyman P.O., J. Biol. Chem 251 (1976) 5457–5463.

[74] Bergeim F.H., Braker W., J. Am. Chem. Soc. 66 (1944) 1459–1460.

[75] Pocker Y., Stone J.T., Biochemistry 6 (1967) 668–678.

[76] Gomaa Z.S., Biomed. Chromatogr. 7 (1993) 134–135.

[77] Bonazzi D., Andrisano V., Di Pietra A.M., Cavrini V., Il Farmaco 49 (1994) 381–386.

[78] Abdine H., Elsayed M.A., Elsayed Y.M., J. Assoc. Off. Anal. Chem. 61 (1978) 695–701.

[79] Forrester R.L., Wataji L.J., Silverman D.A., Pierre K.J., Clin. Chem. 22 (1976) 243–245.