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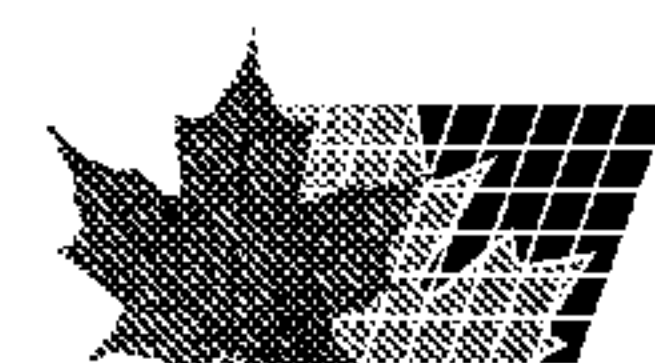
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(54) Titre : INHIBITEURS SPECIFIQUES DE CA IX
(54) Title: CA IX-SPECIFIC INHIBITORS

(57) **Abrégé/Abstract:**

Therapeutic methods for inhibiting the growth of preneoplastic/ neoplastic vertebrate cells that abnormally express MN protein are disclosed. Screening assays are provided for identifying compounds, preferably membraneimpermeant compounds, which inhibit the enzymatic activity of MN protein/ polypeptides and that are useful for treating patients with preneoplastic/neoplastic disease. Further methods are disclosed for the preparation of positively-charged, membrane-impermeant heterocyclic sulfonamide CA inhibitors with high affinity for the membrane-bound carbonic anhydrase CA IX. Preferred CA IX-specific inhibitors are aromatic and heterocyclic sulfonamides, preferably that are membraneimpermeant. Particularly preferred CA IX-specific inhibitors are pyridinium derivatives of such aromatic and heterocyclic sulfonamides. The CA IX-specific inhibitors of the invention can also be used diagnostically/prognostically for preneoplastic/neoplastic disease, and for imaging use, for example, to detect precancerous cells, tumors and/or metastases. The CA IX-specific inhibitors can be labelled or conjugated to radioisotopes for radiotherapy. The CA IX-specific inhibitors may be combined with conventional therapeutic anti-cancer drugs, with other different inhibitors of cancer-related pathways, with bioreductive drugs, or with radiotherapy to enhance the efficiency of each treatment. The CA IX-specific inhibitors may also be combined with CA IX-specific antibodies, preferably monoclonal antibodies or biologically active antibody fragments, more preferably humanized or fully human CA IX monoclonal antibodies or biologically active fragments or such monoclonal antibodies. Still further, the CA IX-specific inhibitors can be used for gene therapy coupled to vectors for targeted delivery to preneoplastic/neoplastic cells expressing CA IX on their surfaces.



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(54) Title: CA IX-SPECIFIC INHIBITORS

(57) **Abstract:** Therapeutic methods for inhibiting the growth of preneoplastic/ neoplastic vertebrate cells that abnormally express MN protein are disclosed. Screening assays are provided for identifying compounds, preferably membraneimpermeant compounds, which inhibit the enzymatic activity of MN protein/ polypeptides and that are useful for treating patients with preneoplastic/neoplastic disease. Further methods are disclosed for the preparation of positively-charged, membrane-impermeant heterocyclic sulfonamide CA inhibitors with high affinity for the membrane-bound carbonic anhydrase CA IX. Preferred CA IX-specific inhibitors are aromatic and heterocyclic sulfonamides, preferably that are membraneimpermeant. Particularly preferred CA IX-specific inhibitors are pyridinium derivatives of such aromatic and heterocyclic sulfonamides. The CA IX-specific inhibitors of the invention can also be used diagnostically/prognostically for preneoplastic/neoplastic disease, and for imaging use, for example, to detect precancerous cells, tumors and/or metastases. The CA IX-specific inhibitors can be labelled or conjugated to radioisotopes for radiotherapy. The CA IX-specific inhibitors may be combined with conventional therapeutic anticancer drugs, with other different inhibitors of cancer-related pathways, with bioreductive drugs, or with radiotherapy to enhance the efficiency of each treatment. The CA IX-specific inhibitors may also be combined with CA IX-specific antibodies, preferably monoclonal antibodies or biologically active antibody fragments, more preferably humanized or fully human CA IX monoclonal antibodies or biologically active fragments or such monoclonal antibodies. Still further, the CA IX-specific inhibitors can be used for gene therapy coupled to vectors for targeted delivery to preneoplastic/neoplastic cells expressing CA IX on their surfaces.



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CA IX-SPECIFIC INHIBITORSFIELD OF THE INVENTION

The present invention is in the general area of medical genetics and in the fields of chemistry, biochemical engineering, and oncology. More specifically, it relates to the use of organic and inorganic compounds, preferably aromatic and heterocyclic sulfonamides, to treat preneoplastic and/or neoplastic diseases by specifically inhibiting the carbonic anhydrase activity of the oncoprotein now known alternatively as the MN protein, the MN/CA IX isoenzyme, the MN/G250 protein or simply MN/CA IX or CA IX or MN. The present invention also relates to methods of treating preneoplastic and/or neoplastic diseases characterized by MN/CA IX overexpression by administering cell membrane-impermeant, inhibitors of MN/CA IX, preferably pyridinium derivatives of aromatic and heterocyclic sulfonamides. The invention further concerns diagnostic/prognostic methods including imaging methods, for preneoplastic/neoplastic diseases, using the disclosed potent CA IX-specific inhibitors, and gene therapy with vectors conjugated to said inhibitors.

BACKGROUND OF THE INVENTION

The instant inventors, Dr. Silvia Pastorekova and Dr. Jaromir Pastorek, with Dr. Jan Zavada ["Zavada et al."], discovered MN/CA IX, a cancer related cell surface protein originally named MN. [73, 123; Zavada et al., U.S. Patent No. 5,387,676 (Feb. 7, 1995).] Zavada et al., WO 93/18152 (published 16 September 1993) and Zavada et al., WO 95/34650 (published 21 December 1995) disclosed the discovery of the MN gene and protein and the strong association of MN gene expression and tumorigenicity led to the creation of methods that are both diagnostic/prognostic and therapeutic for cancer and precancerous conditions. Zavada et al. disclosed further aspects of the MN/CA IX protein and the MN/CA9 gene in Zavada et al., WO 00/24913 (published 4 May 2000).

Zavada et al. cloned and sequenced the MN cDNA and gene, and revealed that MN belongs to a carbonic anhydrase family of enzymes that catalyze the reversible hydration of carbon dioxide to bicarbonate and proton [66, 72]. MN protein (renamed to carbonic anhydrase IX, CA IX) is composed of an extracellular
5 part containing a N-terminal proteoglycan-like region and a catalytically active carbonic anhydrase domain. It is anchored in the plasma membrane by a single transmembrane region and a short intracytoplasmic tail.

Expression of CA IX is restricted to only few normal tissues [74], but is tightly associated with tumors [123]. It is also regulated by cell density *in vitro* [52]
10 and is strongly induced by tumor hypoxia both *in vitro* and *in vivo* [121]. Numerous clinical papers describe the value of CA IX as an indicator of poor prognosis. All CA IX-related studies performed so far support the assumption made in the original Zavada et al., U.S Patent 5,387,676 that CA IX is useful as a diagnostic and/or prognostic tumor marker and as a therapeutic target.

15 MN/CA IX consists of an N-terminal proteoglycan-like domain that is unique among the CAs, a highly active CA catalytic domain, a single transmembrane region and a short intracytoplasmic tail [66, 72, 74, 116]. CA IX is particularly interesting for its ectopic expression in a multitude of carcinomas derived from cervix uteri, ovarian, kidney, lung, esophagus, breast, colon, endometrial, bladder,
20 colorectal, prostate, among many other human carcinomas, contrasting with its restricted expression in normal tissues, namely in the epithelia of the gastrointestinal tract [8, 11, 21, 35, 41, 48, 50, 51, 56, 66, 72, 74, 86, 110, 111, 113, 116, 121, 122].

Uemura et al. [112] reported in 1997 that the G250 antigen was identical to MN/CA IX, years after MN/CA IX had been discovered and sequenced by
25 Zavada et al. {[73, 123]; see also Pastorek et al. [72] and Opavsky et al. [66]}. Uemura et al. [112] stated: "Sequence analysis and database searching revealed that G250 antigen is identical to MN a human tumor-associated antigen identified in cervical carcinoma (Pastorek et al., 1994)."

MN/CA 9 and MN/CA IX – Sequence Similarities

Figure 1A-C shows the full-length MN/CA9 cDNA sequence of 1522 base pairs (bps) [SEQ ID NO: 1], and the full-length MN/CA IX amino acid (aa) sequence of 459 aa [SEQ ID NO: 2]. Figure 2A-F provides the 10,898 bp genomic sequence of MN/CA9 [SEQ ID NO: 3].

Computer analysis of the MN cDNA sequence was carried out using DNASIS*and PROSIS*(Pharmacia*Software packages). GenBank, EMBL, Protein Identification Resource and SWISS-PROT*databases were searched for all possible sequence similarities. In addition, a search for proteins sharing sequence similarities with MN was performed in the MIPS databank with the FastA program [75].

The proteoglycan-like domain [aa 53-111; SEQ ID NO: 4] which is between the signal peptide and the CA domain, shows significant homology (38% identity and 44% positivity) with a keratan sulphate attachment domain of a human large aggregating proteoglycan aggrecan [28].

The CA domain [aa 135-391; SEQ ID NO: 5] is spread over 265 aa and shows 38.9% amino acid identity with the human CA VI isoenzyme [5]. The homology between MN/CA IX and other isoenzymes is as follows: 35.2% with CA II in a 261 aa overlap [63], 31.8% with CA I in a 261 aa overlap [7], 31.6% with CA IV in a 266 aa overlap [65], and 30.5% with CA III in a 259 aa overlap [55].

In addition to the CA domain, MN/CA IX has acquired both N-terminal and C-terminal extensions that are unrelated to the other CA isoenzymes. The amino acid sequence of the C-terminal part, consisting of the transmembrane anchor and the intracytoplasmic tail, shows no significant homology to any known protein sequence.

The MN gene (MN/CA9 or CA9) was clearly found to be a novel sequence derived from the human genome. The overall sequence homology between the cDNA MN/CA9 sequence and cDNA sequences encoding different CA isoenzymes is in a homology range of 48-50% which is considered by ones in the art to be low. Therefore, the MN/CA9 cDNA sequence is not closely related to any CA cDNA sequences.

Very few normal tissues have been found to express MN protein to any significant degree. Those MN-expressing normal tissues include the human gastric

* trade-marks

mucosa and gallbladder epithelium, and some other normal tissues of the alimentary tract. Paradoxically, MN gene expression has been found to be lost or reduced in carcinomas and other preneoplastic/neoplastic diseases in some tissues that normally express MN, e.g., gastric mucosa.

5

CA IX and Hypoxia

Strong association between CA IX expression and intratumoral hypoxia (either measured by microelectrodes, or detected by incorporation of a hypoxic marker pimonidazole, or by evaluation of extent of necrosis) has been demonstrated in the cervical, breast, head and neck, bladder and non-small cell lung carcinomas (NSCLC) [8, 11, 21, 35, 48, 56, 111, 122]. Moreover, in NSCLC and breast carcinomas, correlation between CA IX and a constellation of proteins involved in angiogenesis, apoptosis inhibition and cell-cell adhesion disruption has been observed, possibly contributing to strong relationship of this enzyme to a poor clinical outcome [8]. Hypoxia is linked with acidification of extracellular milieu that facilitates tumor invasion and CA IX is believed to play a role in this process via its catalytic activity [86]. Thus, inhibition of MN/CA IX by specific inhibitors is considered to constitute a novel approach to the treatment of cancers in which CA IX is expressed.

20 CAIs

Teicher et al. [106] reported that acetazolamide – the prototypical CA inhibitor (CAI) – functions as a modulator in anticancer therapies, in combination with different cytotoxic agents, such as alkylating agents; nucleoside analogs; platinum derivatives, among other such agents, to suppress tumor metastasis and to reduce the invasive capacity of several renal carcinoma cell lines (Caki-1, Caki-2, ACHN, and A-498). Such studies demonstrate that CAIs may be used in the management of tumors that overexpress one or more CA isozymes. It was hypothesized that the anticancer effects of acetazolamide (alone or in combination with such drugs) might be due to the acidification of the intratumoral environment ensuing after CA inhibition, although other mechanisms of action of this drug were not excluded [20].

Chegwidden et al. 2001 hypothesized that the *in vitro* inhibition of growth in cell cultures, of human lymphoma cells with two other potent, clinically used sulfonamide

CAls, methazolamide and ethoxzolamide, is probably due to a reduced provision of bicarbonate for nucleotide synthesis (HCO_3^- is the substrate of carbamoyl phosphate synthetase II) as a consequence of CA inhibition [20].

5 All the six classical CAls (acetazolamide, methazolamide, ethoxzolamide, dichlorophenamide, dorzolamide, and dichlorophenamide) used in clinical medicine or as diagnostic tools, show some tumor growth inhibitory properties [18, 78, 101, 102].

The inventors, Dr. Claudia Supuran and Dr. Andrea Scozzafava, reported the design and *in vitro* antitumor activity of several classes of sulfonamide CAls, shown to act as nanomolar inhibitors against the classical isozymes known to possess critical physiological roles, such as CA I, CA II and CA IV. Those compounds were also shown to exert potent inhibition of cell growth in several leukemia, non-small cell lung, ovarian, melanoma, colon, CNS, renal, prostate and breast cancer cell lines, with GI_{50} values of 10 – 75 nM in some cases [77, 91, 92, 15 100].

Wingo et al. reported that three classic sulfonamide drugs (acetazolamide, ethoxzolamide and methoxzolamide) inhibited CA IX carbonic anhydrase activity with values of K_i in the nanomolar range [116]. However, until the present invention, no systematic structure-activity relationship study of sulfonamide inhibition of CA IX, alone or in comparison to other CA isozymes had been performed. 20

Certain pyridinium derivatives of aromatic/heterocyclic sulfonamides have shown nanomolar affinities both for CA II, as well as CA IV, and more importantly, they were unable to cross the plasma membranes *in vivo* [17].

25 Sterling et al. [85] investigated the functional and physical relationship between the downregulated in adenoma bicarbonate transporter and CA II, by using membrane-impermeant sulfonamide inhibitors (in addition to the classical inhibitors such as acetazolamide), which could clearly discriminate between the contribution of the cytosolic and membrane-associated isozymes in these physiological processes.

30

CAS

Carbonic anhydrases (CAs) form a large family of genes encoding zinc metalloenzymes of great physiological importance. As catalysts of reversible hydration of carbon dioxide, these enzymes participate in a variety of biological processes, including respiration, calcification, acid-base balance, bone resorption, formation of aqueous humor, cerebrospinal fluid, saliva and gastric acid [reviewed in Dodgson et al. (27)]. CAs are widely distributed in different living organisms. In higher vertebrates, including humans, 14 different CA isozymes or CA-related proteins (CARP) have been described, with very different subcellular localization and tissue distribution [40, 93, 95, 94, 102]. Basically, there are several cytosolic forms (CA I-III, CA VII), four membrane-bound isozymes (CA IV, CA IX, CA XII and CA XIV), one mitochondrial form (CA V) as well as a secreted CA isozyme, CA VI [40, 93, 94, 95, 102].

It has been shown that some tumor cells predominantly express only some membrane-associated CA isozymes, such as CA IX and CA XII [2, 67, 68, 78, 87, 93, 95]. Occasionally, nuclear localization of some isoenzymes has been noted [64, 69, 70]. Not much is presently known about the cellular localization of the other isozymes.

CAs and CA-related proteins show extensive diversity in their tissue distribution, levels, and putative or established biological functions [105]. Some of the CAs are expressed in almost all tissues (CA II), while the expression of others appears to be more restricted (e.g., CA VI and CA VII in salivary glands [32, 69, 71]. The CAs and CA-related proteins also differ in kinetic properties and susceptibility to inhibitors [82].

Most of the clinically used sulfonamides mentioned above are systemically acting inhibitors showing several undesired side effects due to inhibition of many of the different CA isozymes present in the target tissue/organ (14 isoforms are presently known in humans) [93, 94, 95, 102]. Therefore, many attempts to design and synthesize new sulfonamides were recently reported, in order to avoid such side effects [13, 17, 42, 62, 80, 99, 100]. At least four CA isozymes (CA IV, CA IX, CA XII and CA XIV) are associated to cell membranes, with the enzyme active site generally oriented extracellularly [93, 94, 95, 102]. Some of these isozymes

were shown to play pivotal physiological roles (such as for example CA IV and XII in the eye, lungs and kidneys, CA IX in the gastric mucosa and many tumor cells) [3, 18, 22, 29, 49, 67, 68, 83, 93, 94, 95, 102], whereas the function of other such isozymes (CA XIV) is for the moment less well understood [93, 95]. Due to the extracellular
5 location of these isozymes, if membrane-impermeant CA inhibitors (CAIs) could be designed, only membrane-associated CAs would be affected.

The first approach towards introducing the membrane-impermeability to CAIs from the historical point of view was that of attaching aromatic/heterocyclic sulfonamides to polymers, such as polyethyleneglycol, aminoethyl-dextran, or dextran
10 [39, 60, 107]. Such compounds, possessing molecular weights in the range of 3.5 - 99 kDa, prepared in that way, showed indeed membrane-impermeability due to their high molecular weights, and selectively inhibited *in vivo* only CA IV and not the cytosolic isozymes (primarily CA II), being used in several renal and pulmonary physiological studies [39, 60, 107]. Due to their macromolecular nature, such
15 inhibitors could not be developed as drugs/diagnostic tools, since *in vivo* they induced potent allergic reactions [39, 60, 93, 95, 107]. A second approach for achieving membrane-impermeability is that of using highly polar, salt-like compounds. Only one such sulfonamide has until recently been used in physiological studies, QAS (quaternary ammonium sulphanilamide), which has been reported to inhibit
20 only extracellular CAs in a variety of arthropods (such as the crab *Callinectes sapidus*) and fish [57]. The main draw-back of QAS is its high toxicity in higher vertebrates [57].

Enzyme activity of carbonic anhydrases (including that of CA IX) can be efficiently blocked by sulfonamide inhibitors. That fact has been therapeutically
25 exploited in diseases caused by excessive activities of certain CA isoforms (e.g. CA II in glaucoma). There is also an experimental evidence that sulfonamides may block tumor cell proliferation and invasion *in vitro* and tumor growth *in vivo*, but the targets of those sulfonamides have not been identified yet. However, the sulfonamides available so far indiscriminately inhibit various CA isoenzymes (14 are presently
30 known in humans) that are localized in different subcellular compartments and play diverse biological roles. This lack of selectivity compromises the clinical utilization of these compounds (due to undesired side effects caused by concurrent inhibition of

many CA isoforms) and represents a main drawback also for the sulfonamide application against CA IX in anticancer therapy.

Thus, there is a need in the art for membrane-impermeant, potent CA IX inhibitors, which would become doubly selective inhibitors for CA IX. The
5 inventors have previously made and described some of the membrane-impermeant molecules described here; however, they were characterized only for their ability to inhibit CA I, CA II and CA IV. While others have studied effects of selective inhibition of extracellular CA by membrane impermeant agents in retinal pigmented epithelia or muscle [34, 120], these agents have not been characterized for their ability to
10 inhibit CA IX. Since CA IX is one of the few extracellular carbonic anhydrases, a membrane-impermeant selective inhibitor of CA IX would be doubly selective for this enzyme and thereby avoid side effects associated with nonspecific CA inhibition.

SUMMARY OF THE INVENTION

15 The inventors approached the problem of lack of selectivity of CAls by taking advantage of features that distinguish CA IX from the other CA isoforms. First of all, CA IX is an integral plasma membrane protein with an active site exposed on the extracellular side. In this respect, it is similar to some CAs (CA IV, CA XII and CA XIV) but differs from all other isoforms. Among these membrane-bound isoenzymes,
20 CA IX shows some differences in the amino acid sequence of the catalytic domain that may influence the topology of the active site cavity and hence the interaction with sulfonamides. In addition, unlike the other CA isoforms, CA IX is expressed preferentially in hypoxic areas of tumors with poor prognosis.

The inventors evaluated inhibition profiles of CA IX with a series of
25 aromatic and heterocyclic compounds and found that some of them inhibit CA IX more efficiently than the other widely distributed isoforms CA I, II and IV. Several nanomolar CA IX inhibitors have been detected both among the aromatic and the heterocyclic compounds. This finding is very promising for the design of CA IX-specific inhibitors by modification of their physico-chemical properties such as
30 charge, size and bioreductivity to conform the characteristic properties of CA IX.

The inventors found that some of the more bulky compounds that strongly inhibited CA IX were very weak inhibitors of CA I, II and IV, possibly due to the fact that the CA IX active site cavity is larger than that of the other investigated isoenzymes. The compounds of such type, identified by screening as disclosed
5 herein, based on the selective inhibition of tumor-associated isoform CA IX may be particularly preferred CA IX specific inhibitors, that could be used in new anticancer therapies and in the diagnostic/prognostic methods of this invention.

The inventors have shown that CA IX is capable of reducing E-cadherin-mediated cell-cell adhesion that may be important for increased invasion
10 capacity of the cells [103]. CA IX was found by the inventors also to contribute to acidification of extracellular pH in hypoxia but not in normoxia (*unpublished data*). The latter result indicates that hypoxia up-regulates both expression level and enzyme activity of CA IX, that is, hypoxia activates the CA catalytic activity of CA IX. That is a very important finding because intratumoral hypoxia is a clinically relevant
15 factor increasing aggressiveness of tumor cells and reducing success of therapy. Hypoxia is usually accompanied by acidification of extracellular microenvironment, which facilitates tumor invasion and metastasis. CA IX appears to participate in this phenomenon by catalyzing hydration of carbon dioxide to generate bicarbonate ions that are then transported into cell interior and protons that acidify extracellular pH.
20 Therefore, inhibition of the CA IX catalytic activity resulting in reduced extracellular acidification may have direct anticancer effects or may modulate efficiency of those conventional chemotherapeutic drugs whose uptake is pH-dependent.

The instant invention is related to (1) the recognition that certain carbonic anhydrase inhibitors (CAIs), preferably sulfonamides, selectively target the
25 cancer-related, hypoxia-induced MN/CA IX; (2) the use of such CAIs, preferably sulfonamides, as lead compounds for the design and synthesis of MN/CA IX-specific inhibitors; (3) the employment of said MN/CA IX-specific inhibitors for anticancer therapy based upon the inhibition of MN/CA IX-mediated acidification of tumor microenvironments; and (4) the use of the specificity of potent MN/CA IX-specific
30 inhibitors for diagnostic/prognostic methods including imaging methods, such as scintigraphy, and for gene therapy. The invention is particularly directed to the use of CA IX-specific inhibitors for the development of drugs possessing anticancer

properties and to modulate conventional chemotherapy for preneoplastic and neoplastic disease characterized by CA IX expression, particularly CA IX overexpression.

In one aspect, the invention concerns methods of treating a mammal for a pre-cancerous or cancerous disease, wherein said disease is characterized by overexpression of MN/CA IX protein, comprising administering to said mammal a therapeutically effective amount of a composition comprising a compound, wherein said compound is selected from the group consisting of organic and inorganic molecules, and wherein said compound is determined to be a potent inhibitor of MN/CA IX enzymatic activity in a screening assay comprising:

a) preparing serial dilutions of said compound and serial dilutions of MN/CA IX protein or a fragment of the MN/CA IX protein that comprises the carbonic anhydrase domain;

b) preincubating a dilution of said compound with a dilution of said MN/CA IX protein or said MN/CA IX protein fragment for ten minutes at 20°C;

c) combining said preincubated mixture of said diluted compound and said diluted MN/CA IX protein or protein fragment with a substrate, consisting essentially of a saturated CO₂ solution, phenol red to 0.2mM, Na₂SO₄ to 0.1M, and Hepes buffer (pH 7.5) to 10mM, in a reaction vessel for a period of 10 to 100 seconds at 20°C;

d) concurrently measuring the optical density, at the absorbance maximum of 557 nm, of the contents of said reaction vessel, using a stopped flow spectrophotometer; and

e) determining the inhibition constant K_i of said compound;

wherein if said inhibition constant K_i is determined to be less than about 50 nanomolar, said compound is determined to be a potent inhibitor of MN/CA IX enzymatic activity; and wherein said compound is not selected from the group consisting of acetazolamide, ethoxzolamide, methazolamide and cyanate. Said mammal is preferably human, and said K_i is preferably less than about 35 nanomolar, more preferably less than about 25 nanomolar, and still more preferably less than about 10 nanomolar.

Such methods can also be framed as methods of treating precancer and/or cancer, or inhibiting the growth of precancerous and/or cancerous cells in a mammalian subject, wherein said precancer and cancer are characterized by the overexpression of MN/CA IX. Said methods can also be framed as inhibiting the growth of such precancerous or cancerous mammalian cells overexpressing MN/CA IX comprising contacting said cells with a CA IX-specific inhibitor of this invention.

The CA IX-specific inhibitors of this invention can be administered in a therapeutically effective amount, preferably dispersed in a physiologically acceptable nontoxic liquid vehicle. Different routes of administration may be preferred depending on the site or type of preneoplastic/neoplastic disease, for example, solid or non-solid tumor or metastasis. In general, parenteral administration would be preferred to avoid undesired effects of systemic treatment, for example, those that could be occasioned by binding of the inhibitors to the gastrointestinal mucosa. Injection into or into the vicinity of the preneoplastic/neoplastic disease would be generally preferred. For example, such injections could be intravenous, intraperitoneal, rectal, subcutaneous, intramuscular, intraorbital, intracapsular, intraspinal, intrasternal, intramedullary, intralesional, intradermal, among other routes of injection. Also, other modes of administration, for example, by suppository or topically, can be used as would be appropriate to the target disease. The pharmaceutical formulation would be designed in accordance with known standards as suitable for the route of administration.

Said CA IX-specific inhibitors are preferably organic, more preferably aromatic or heterocyclic, and still more preferably an aromatic sulfonamide or a heterocyclic sulfonamide. Said aromatic sulfonamide may be a substituted aromatic sulfonamide, wherein said aromatic sulfonamide comprises an aromatic ring structure bearing a sulfonamide moiety bonded to said ring structure and optionally bearing one or more substituents independently selected from the group consisting of halogeno, nitro, and an alkylamino group, wherein the alkyl radical of said alkylamino group comprises 1 to 4 carbon atoms.

Preferably the CA IX-specific inhibitors of this invention are more potent inhibitors of MN/CA IX enzymatic activity than of the enzymatic activity of a carbonic anhydrase selected from the group consisting of CA I, CA II and CA IV.

More preferably, the CA IX-specific inhibitors are more potent inhibitors of MN/CA IX enzymatic activity than of the enzymatic activity of at least two carbonic anhydrases selected from the group consisting of CA I, CA II and CA IV. Still more preferably, the CA IX-specific inhibitors are more potent inhibitor of MN/CA IX enzymatic activity
5 than of the enzymatic activity of each of the carbonic anhydrases in the group consisting of CA I, CA II and CA IV.

However, since CA II is a particularly abundant and significant CA, that is cytosolic, it is important when the CA IX-specific inhibitors of this invention are not membrane-impermeant, that they may be more potent inhibitors of MN/CA IX
10 enzymatic activity than of the enzymatic activity of CA II. A method comprising the following steps provides an exemplary screening assay that can be used to determine the K_i of a compound in inhibiting the enzymatic activity of CA II:

a) preparing serial dilutions of said compound and serial dilutions of CA II;

15 b) preincubating a dilution of said compound with a dilution of CA II for ten minutes at 20°C;

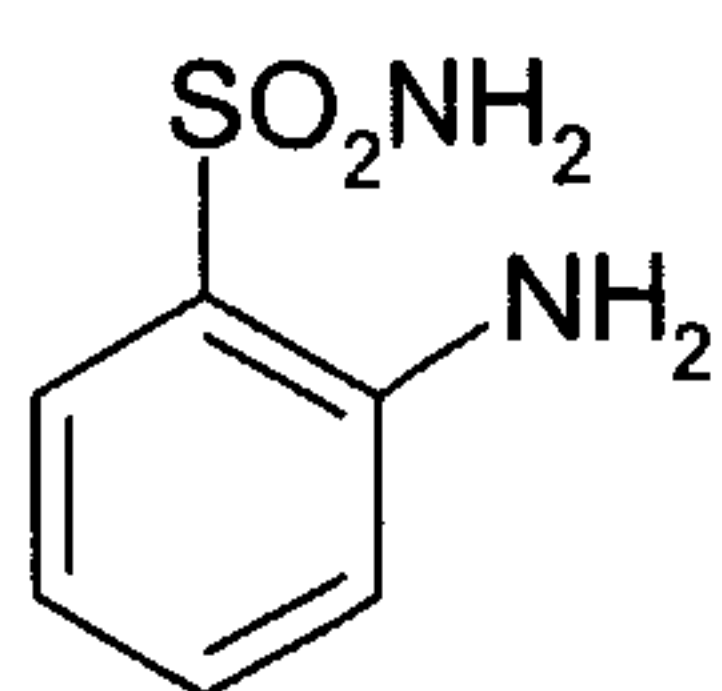
c) combining said preincubated mixture of said compound and said CA II with a substrate solution, consisting essentially of 4-nitrophenylacetate in anhydrous acetonitrile, in a reaction vessel for a period of 1 to 3 minutes at 25°C;

20 d) concurrently measuring the optical density, at the absorbance maximum of 400 nm, of the contents of said reaction vessel, using a spectrophotometer; and

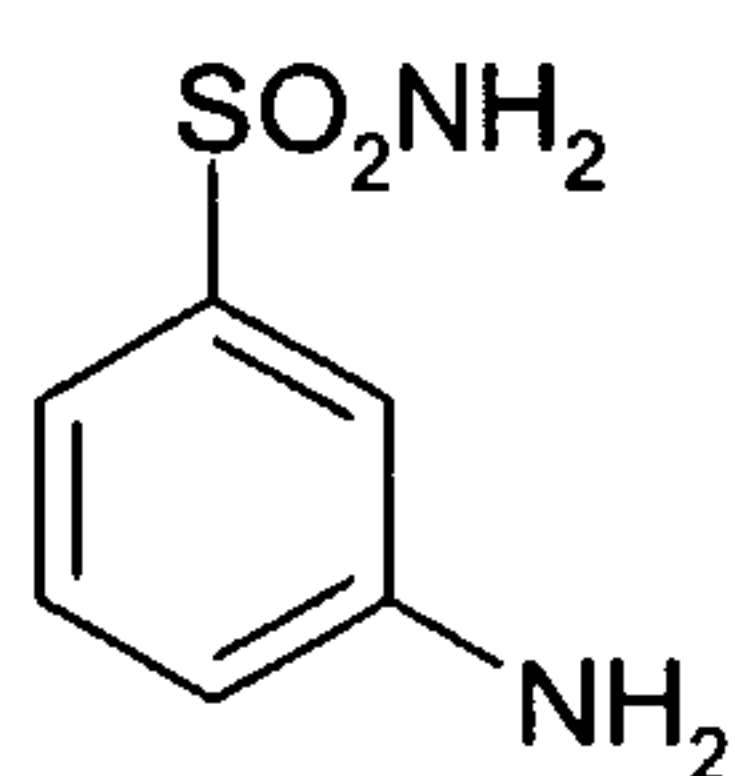
e) determining the inhibition constant K_i of said compound.

Exemplary and preferred aromatic sulfonamide or heterocyclic

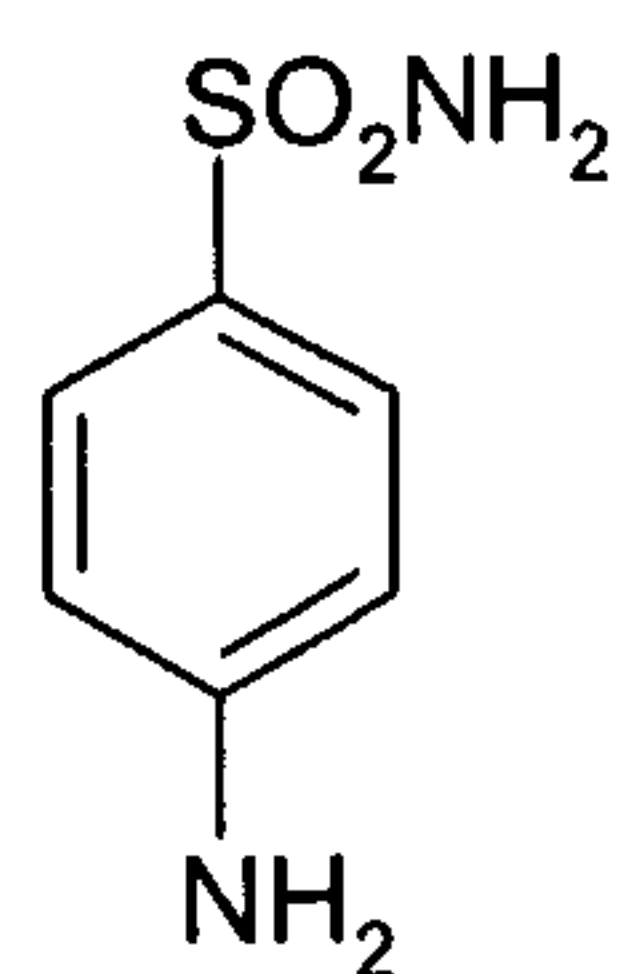
25 sulfonamide CA IX-specific inhibitors of this invention are selected from the group consisting of:



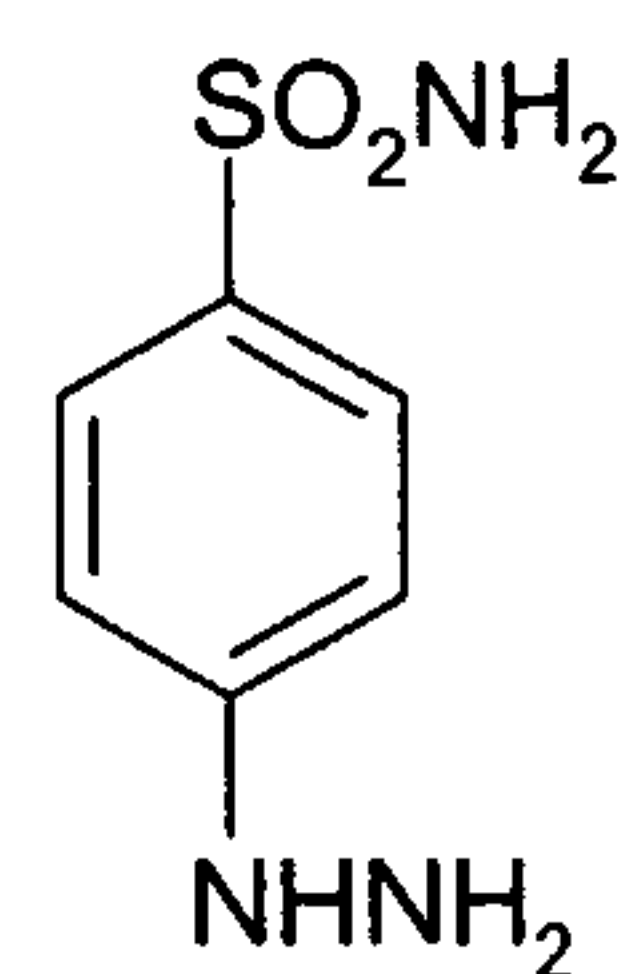
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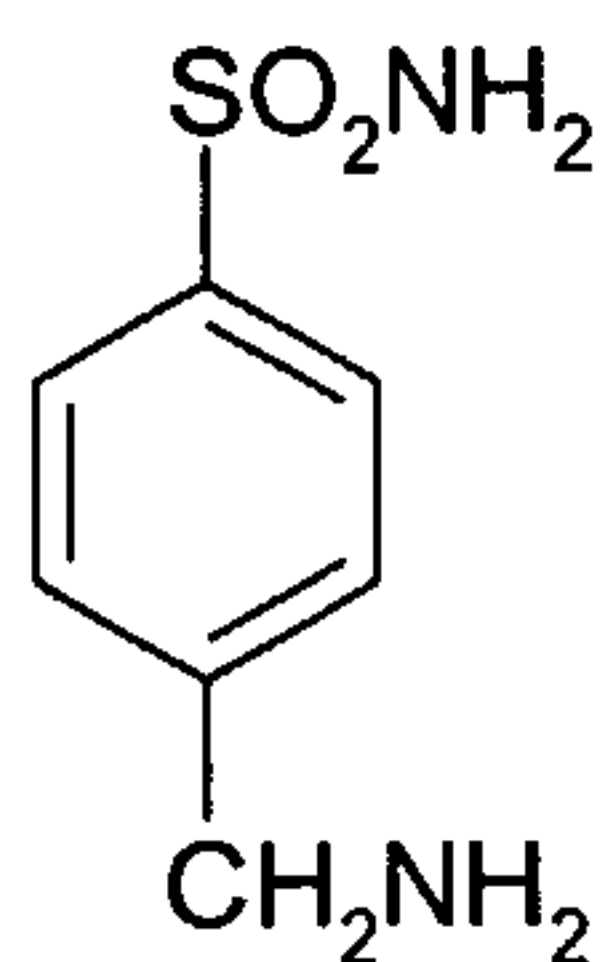
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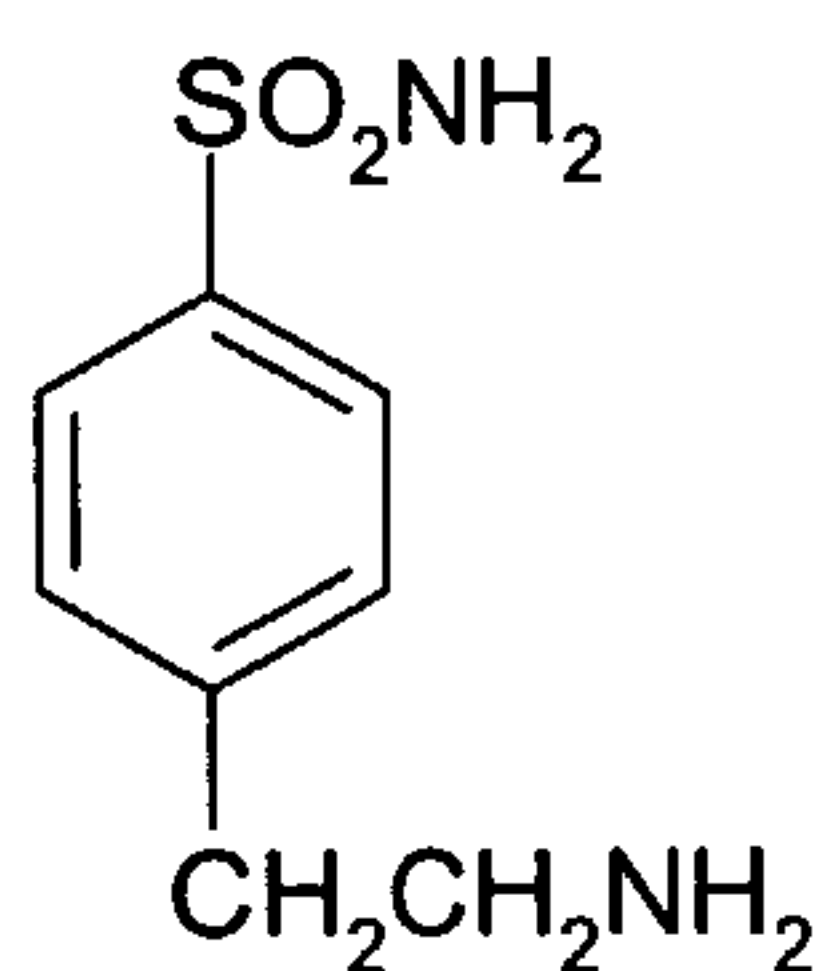
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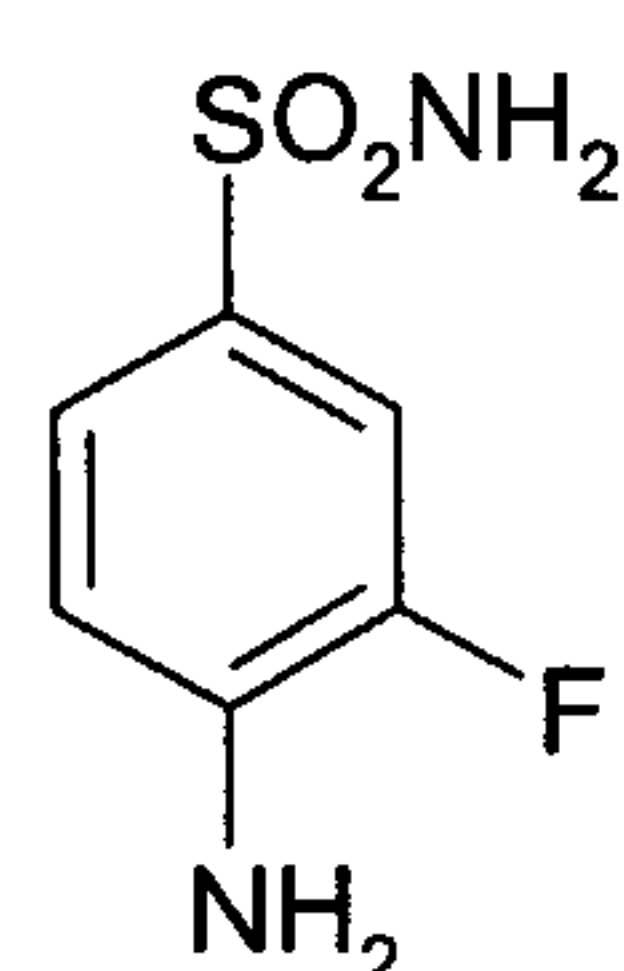
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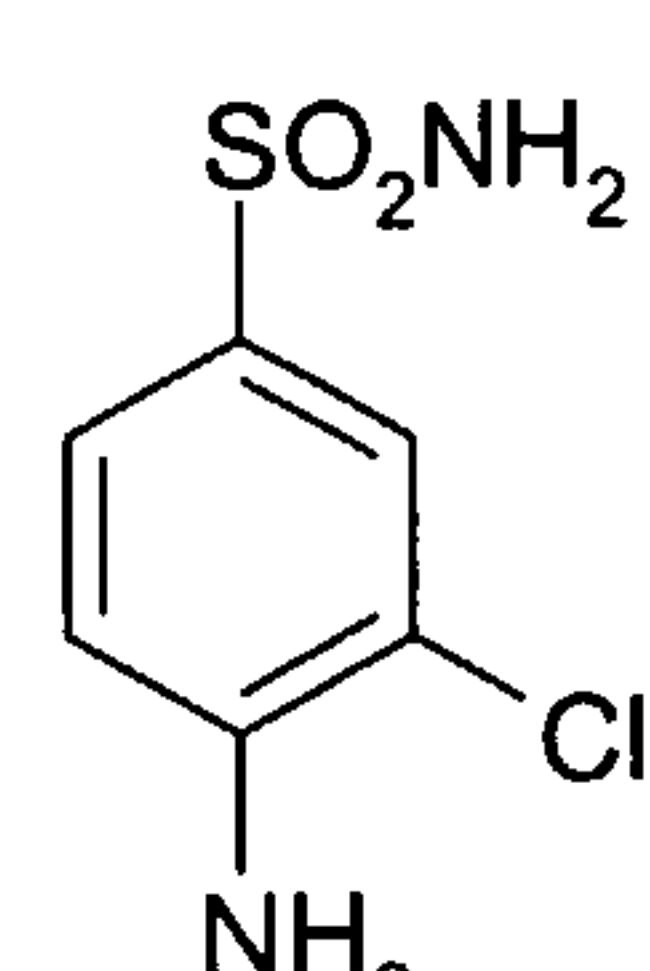
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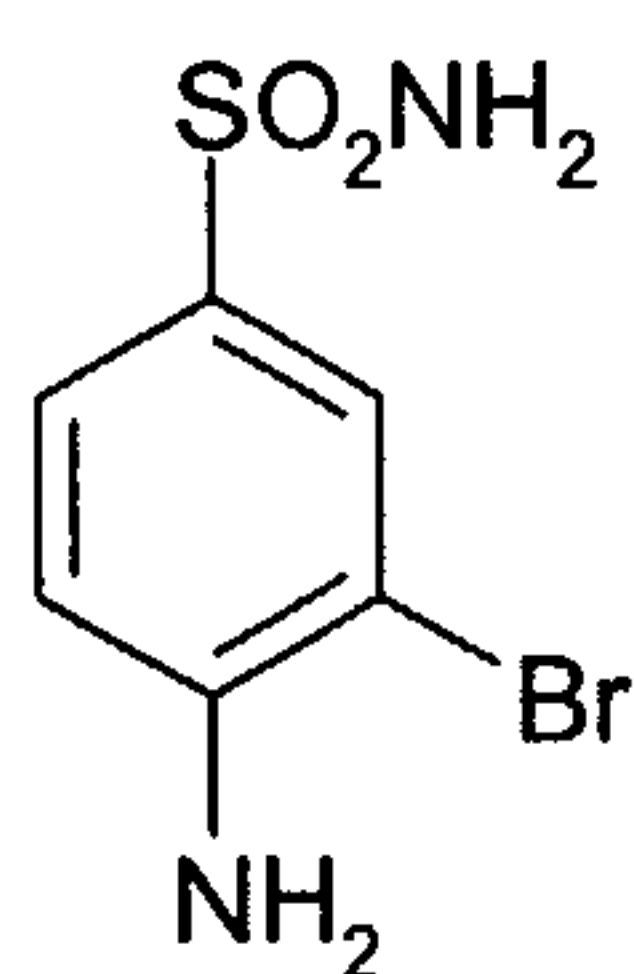
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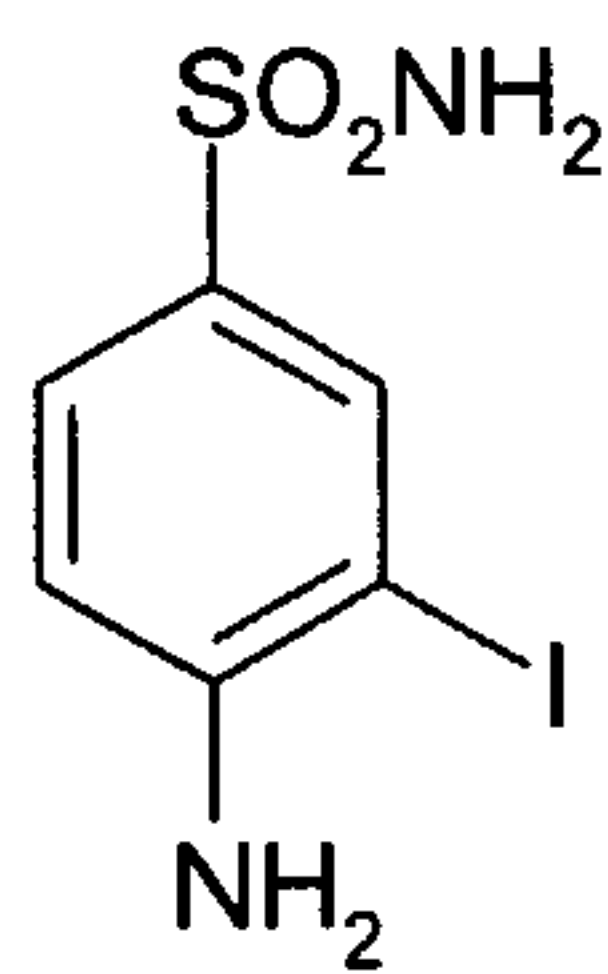
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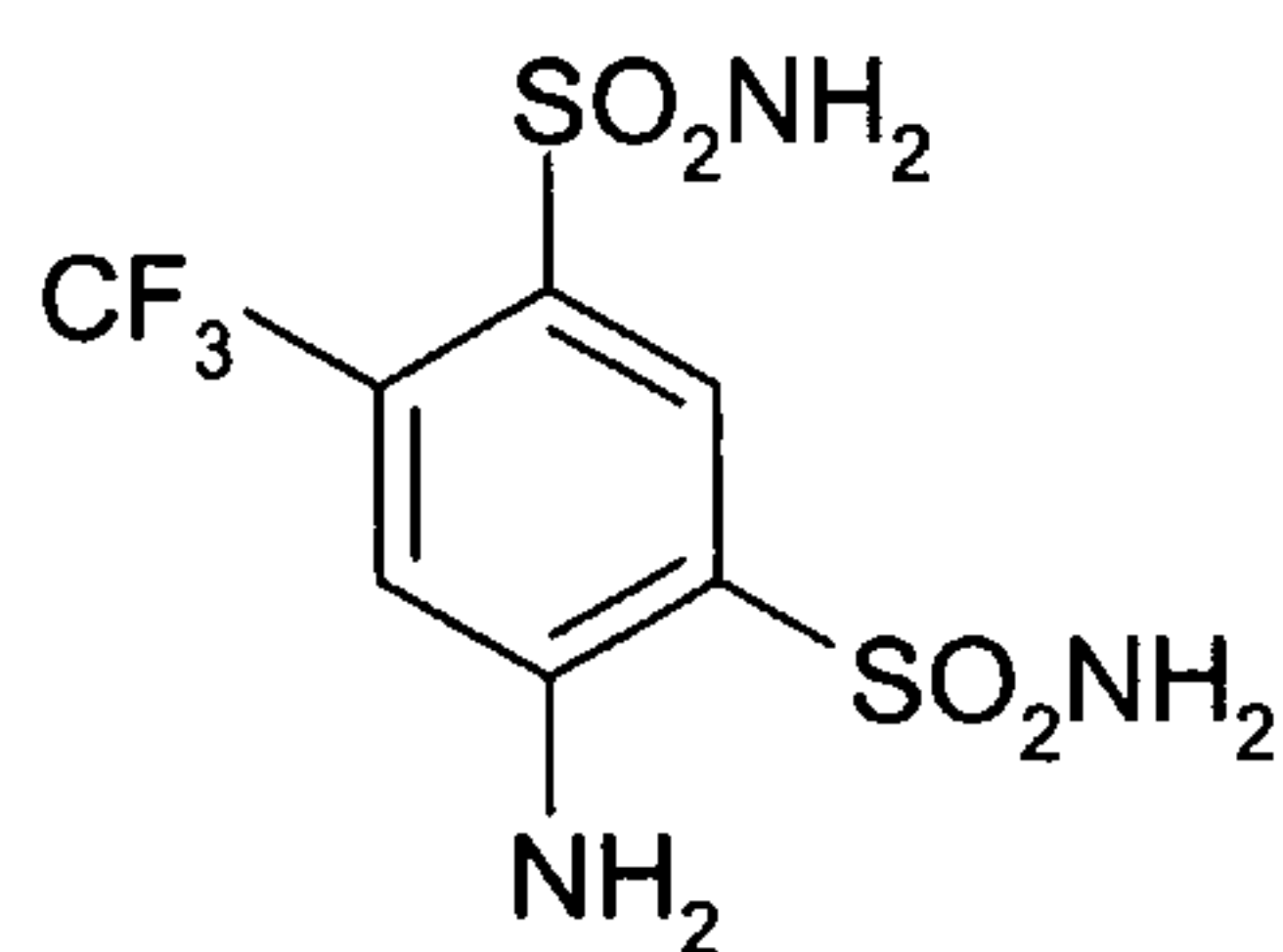
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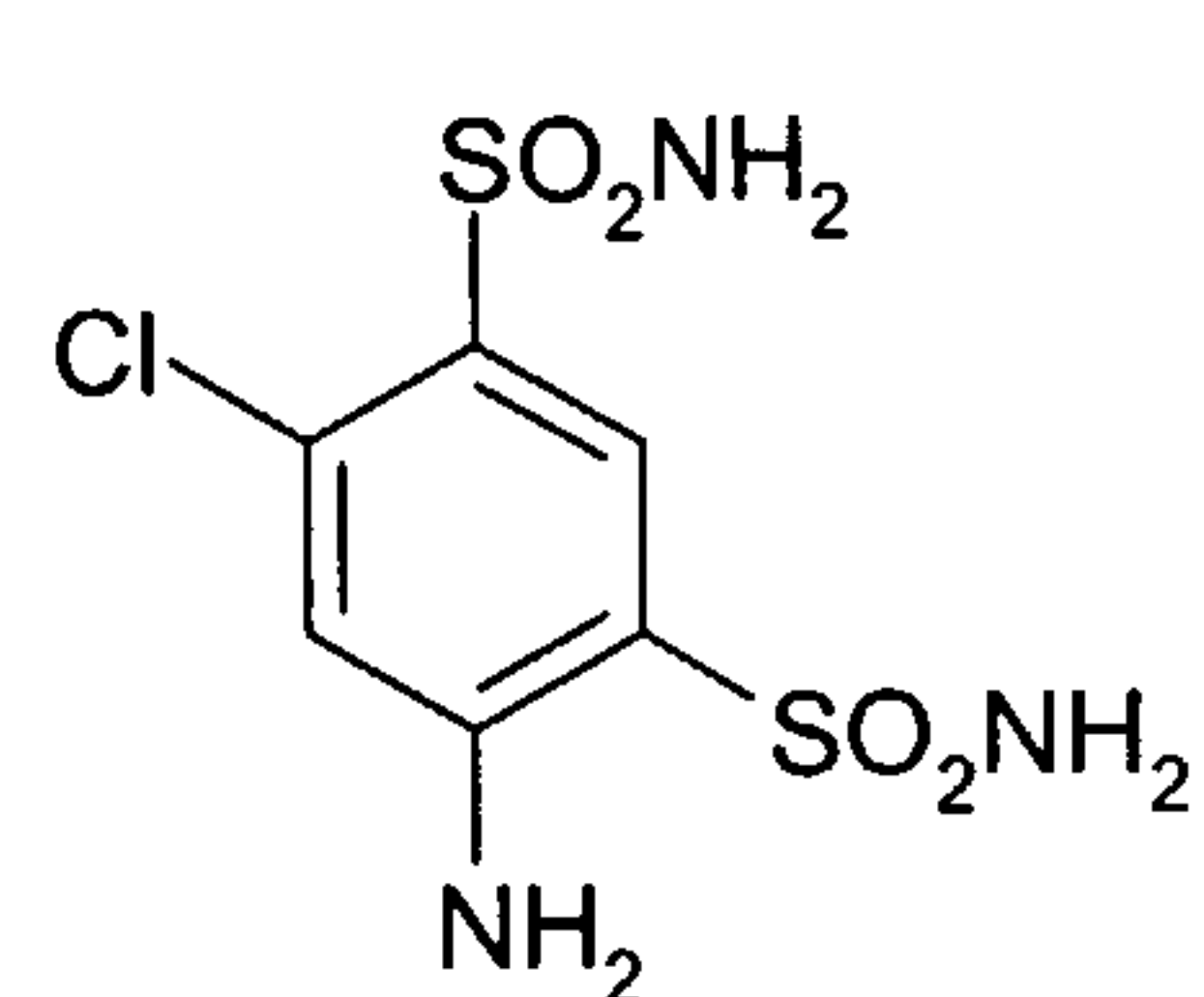
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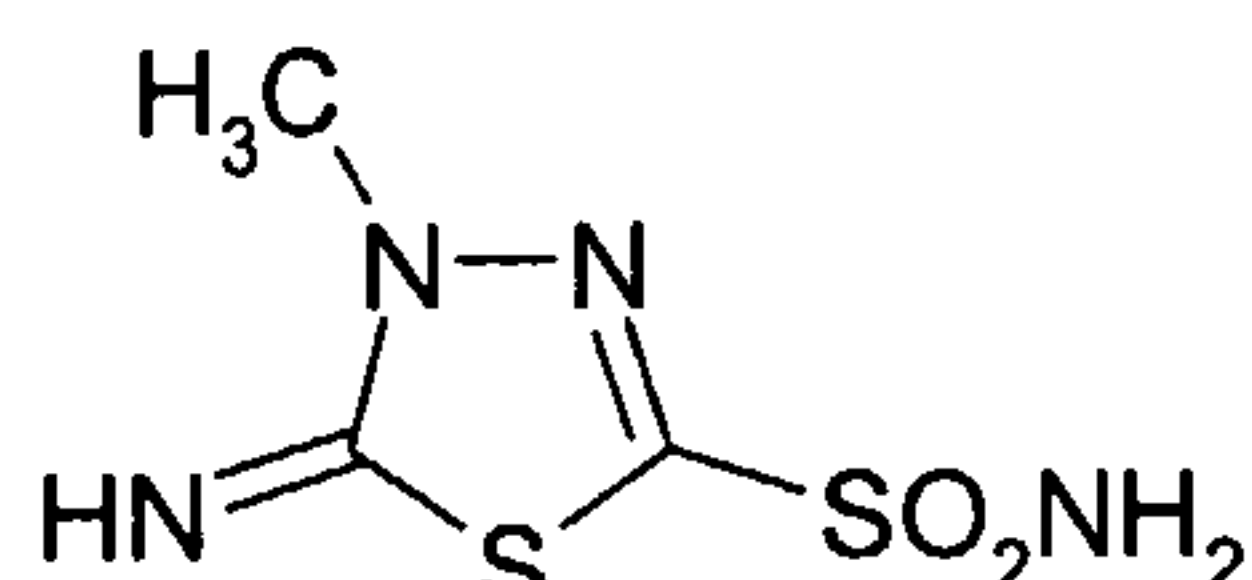
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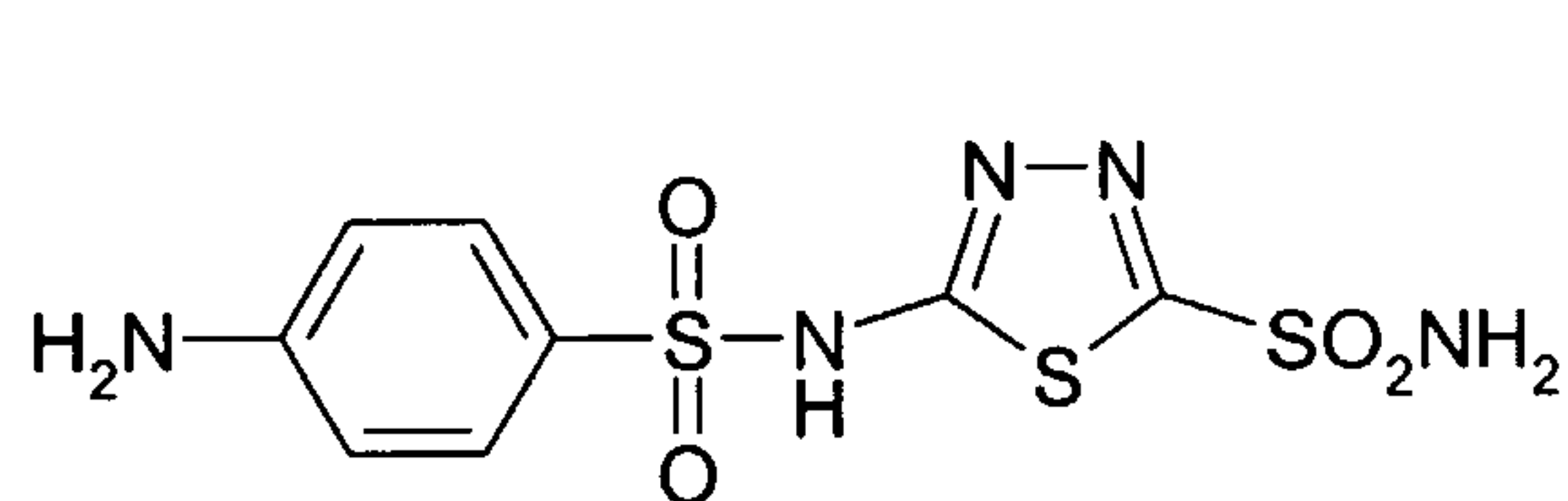
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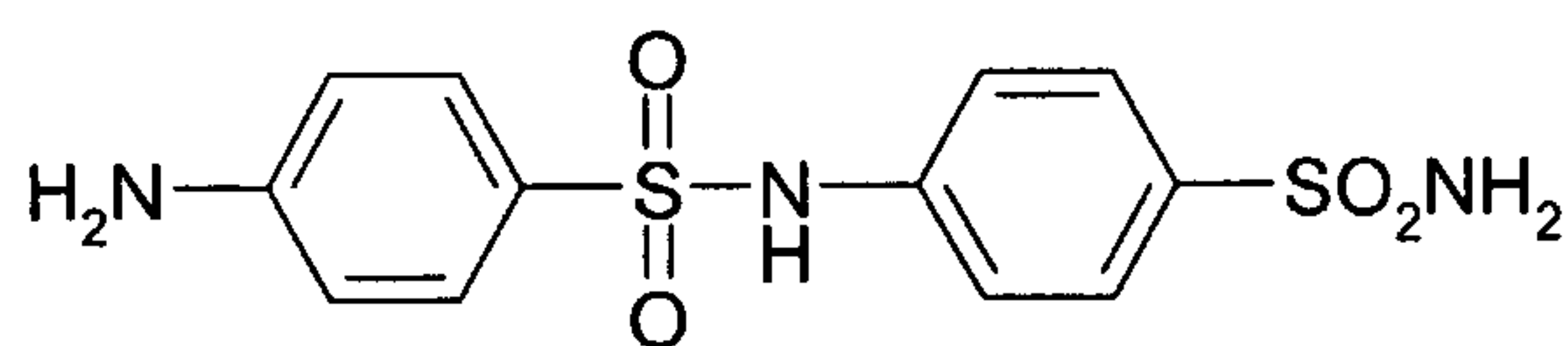
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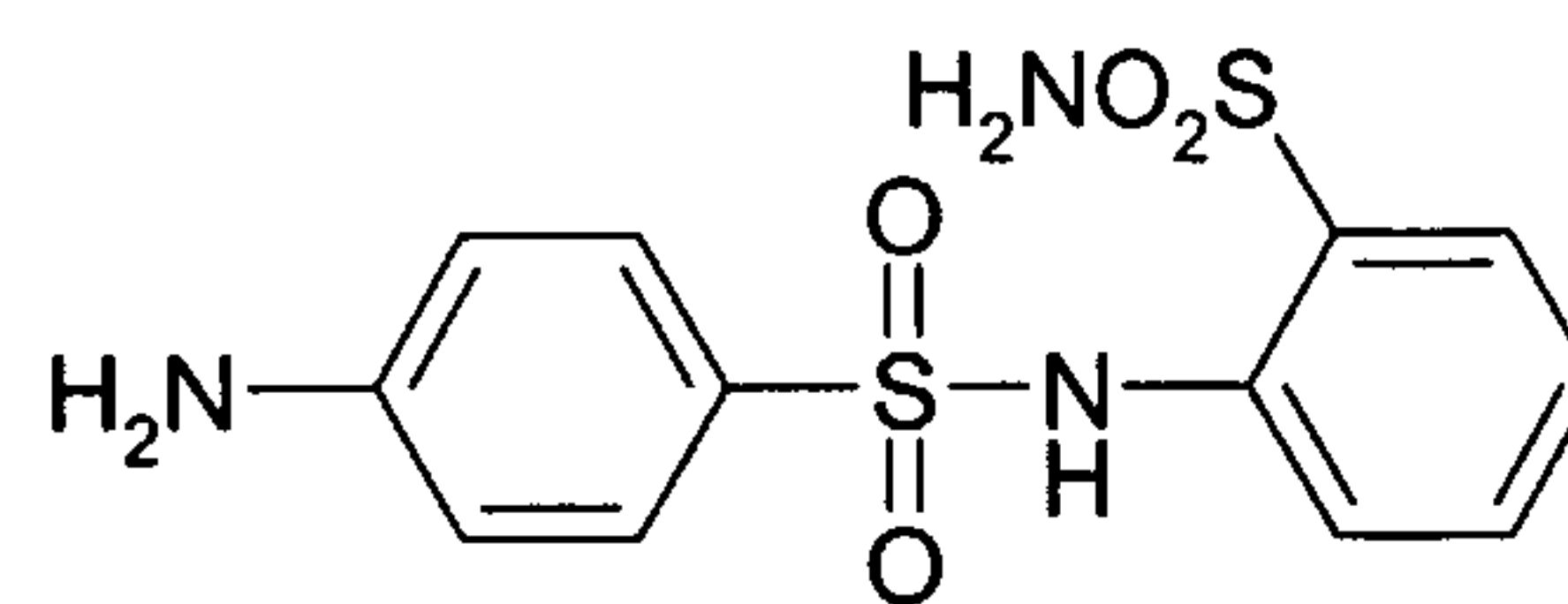
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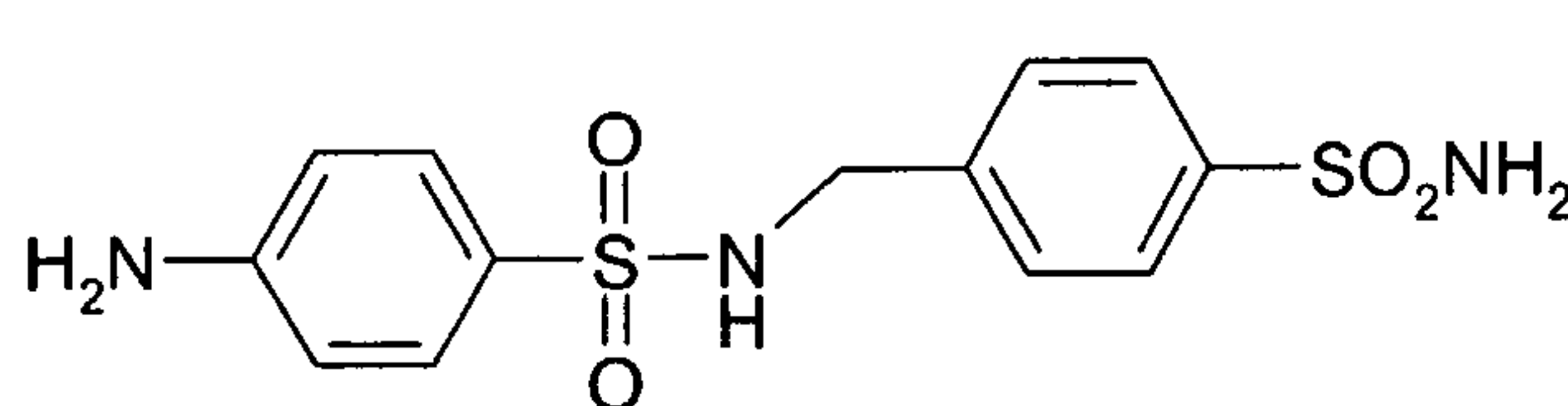
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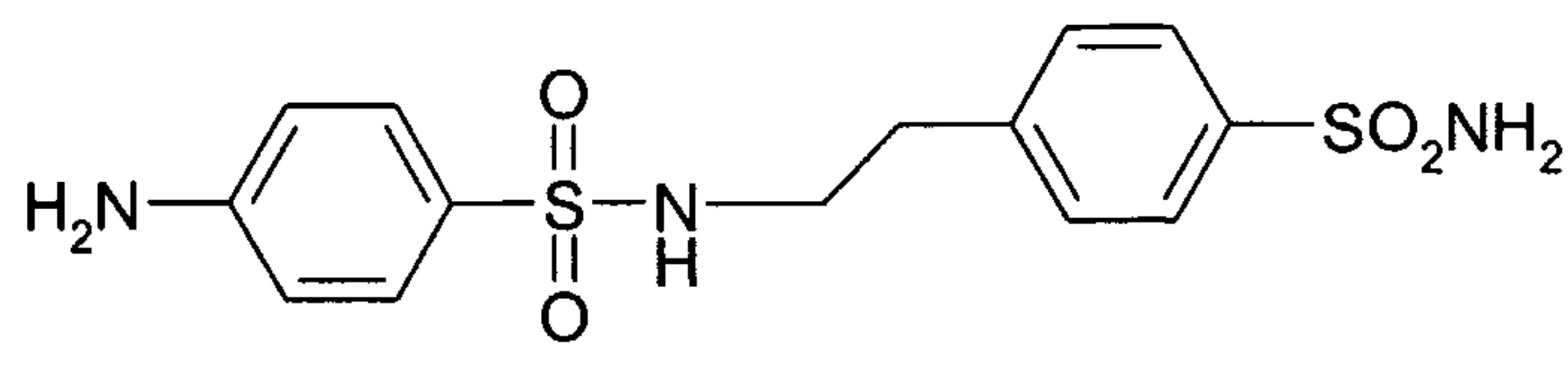
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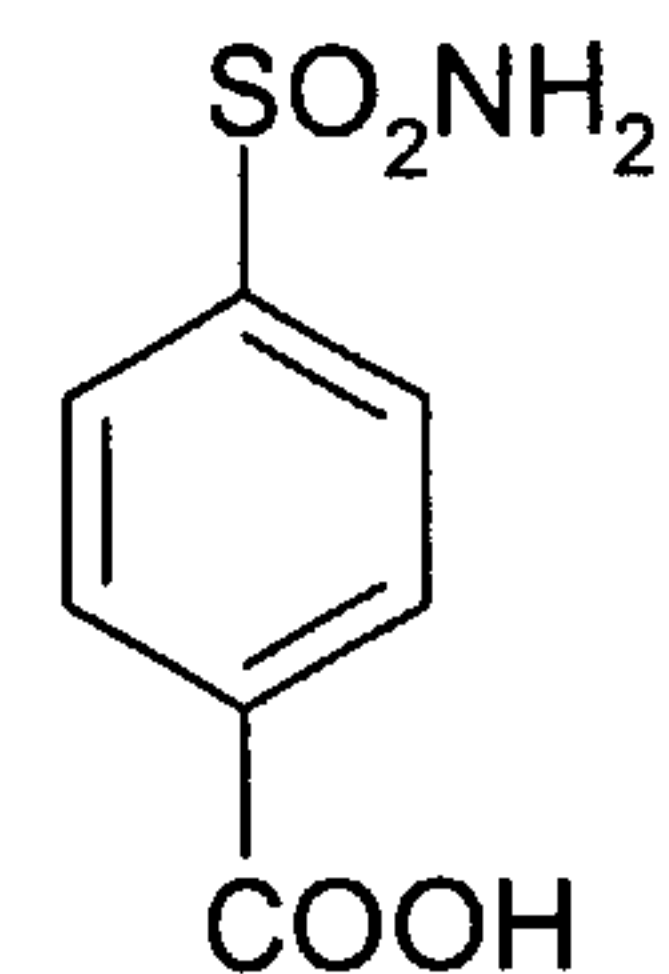
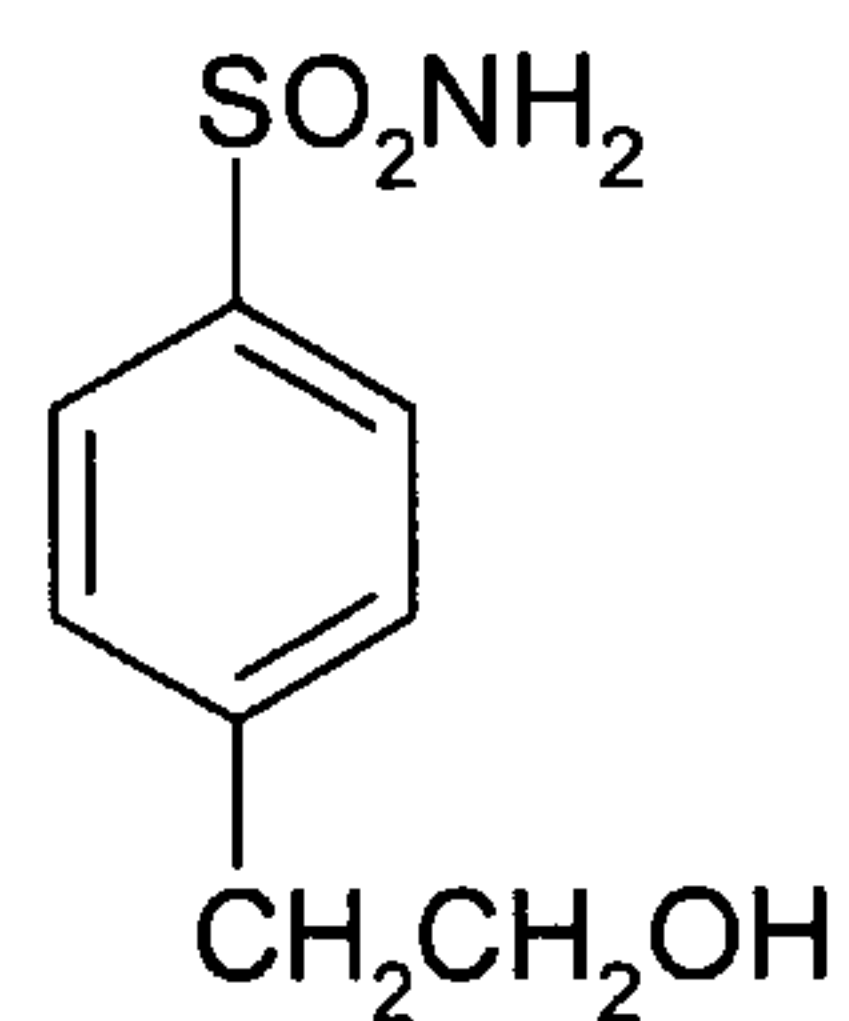
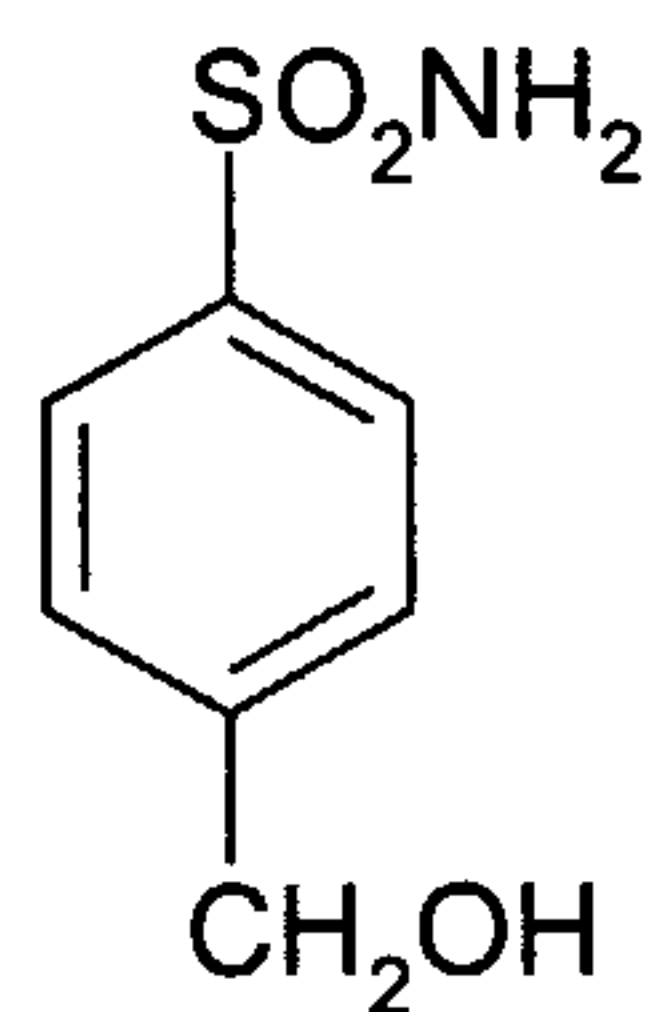
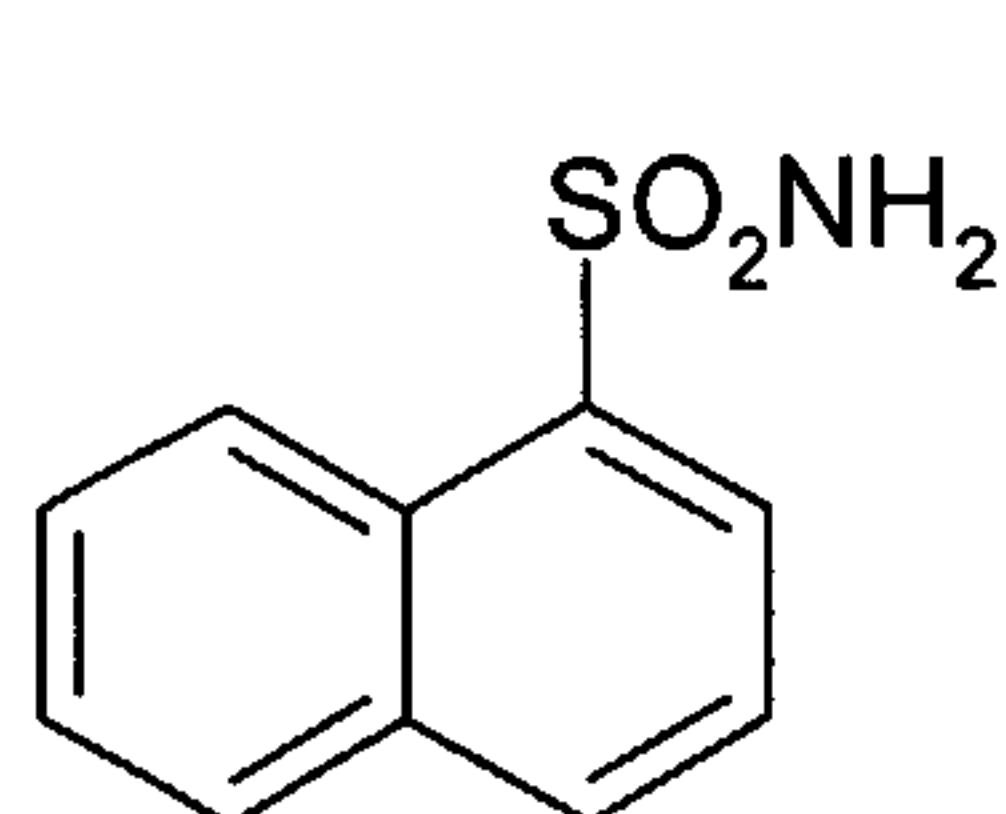
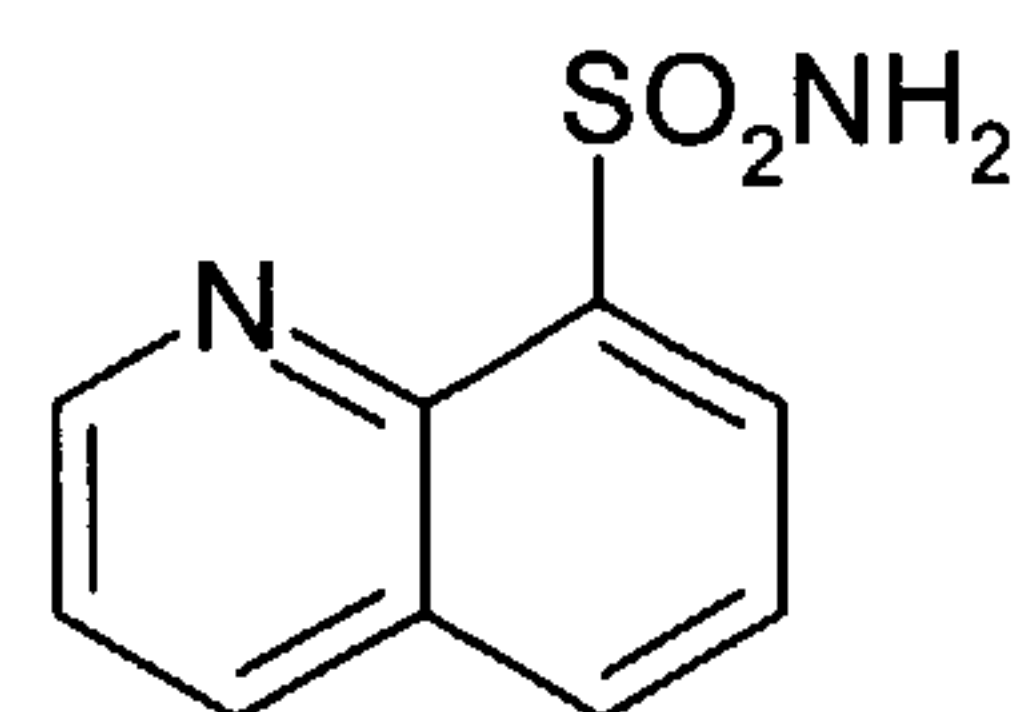
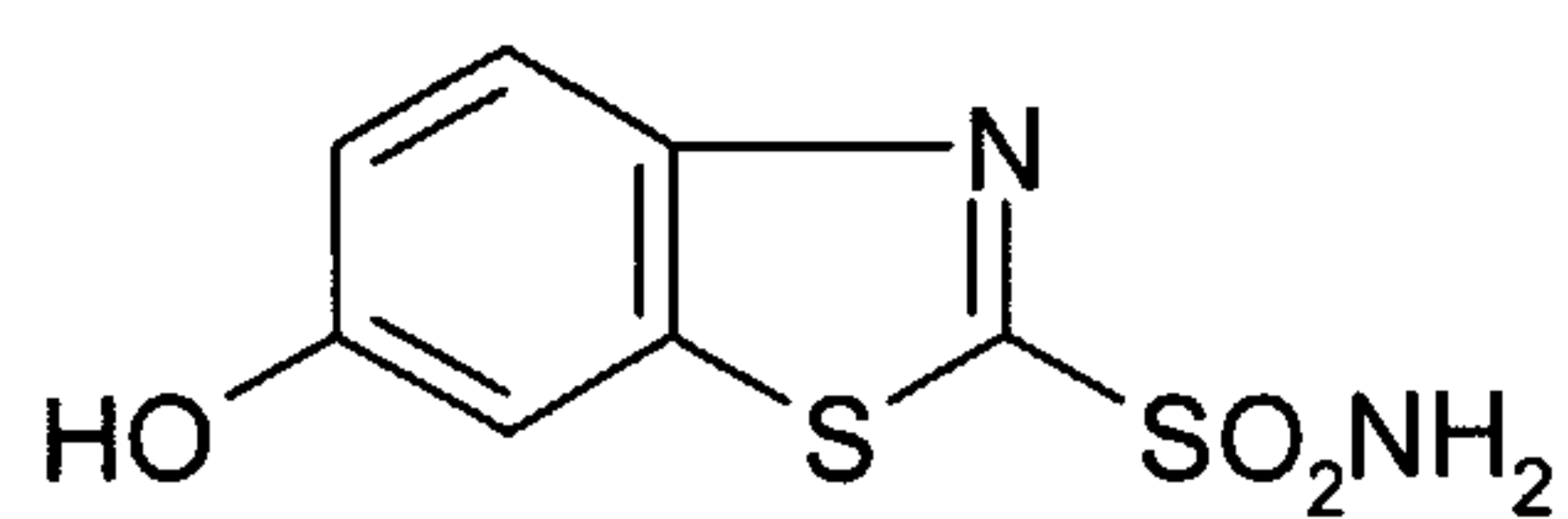
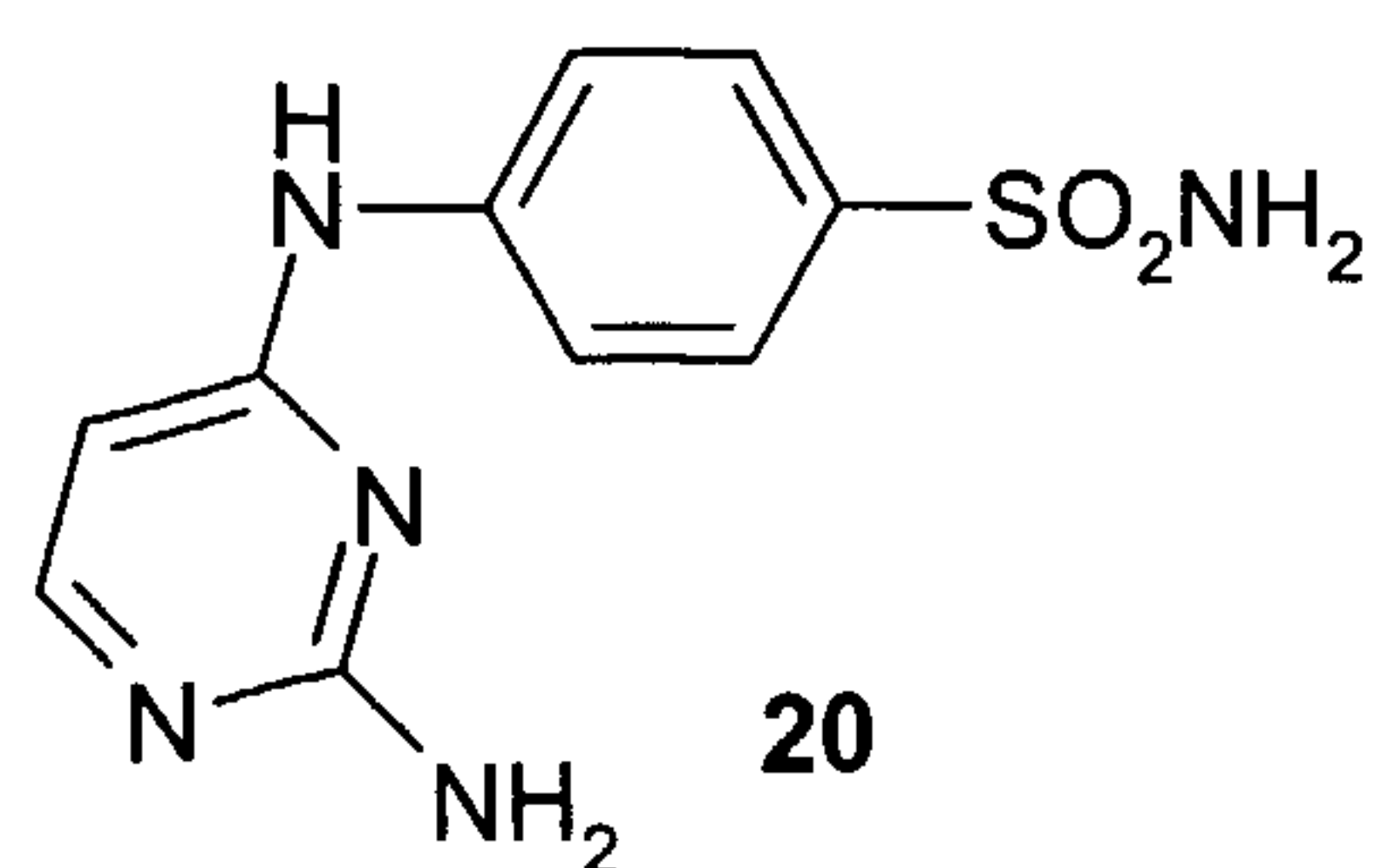
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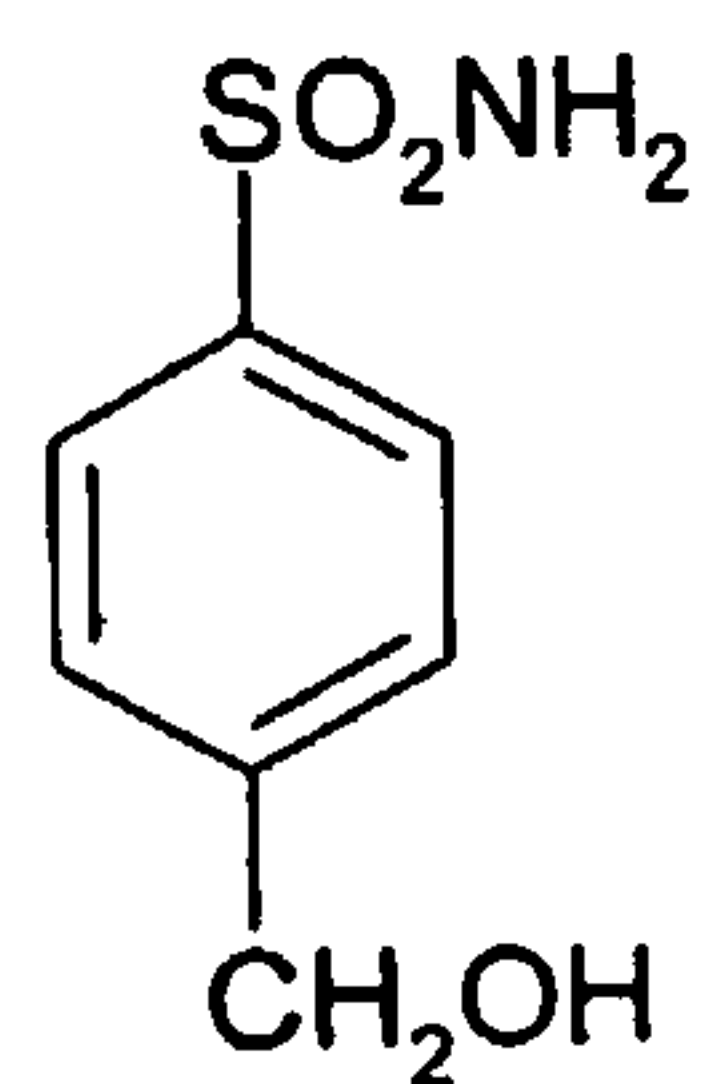
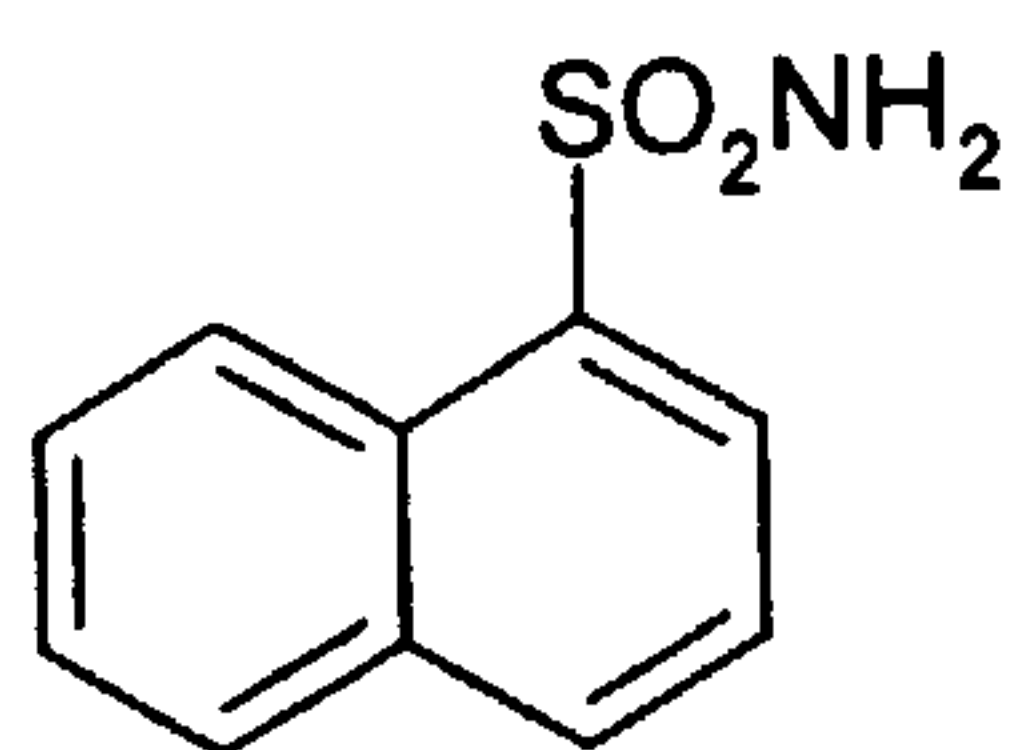
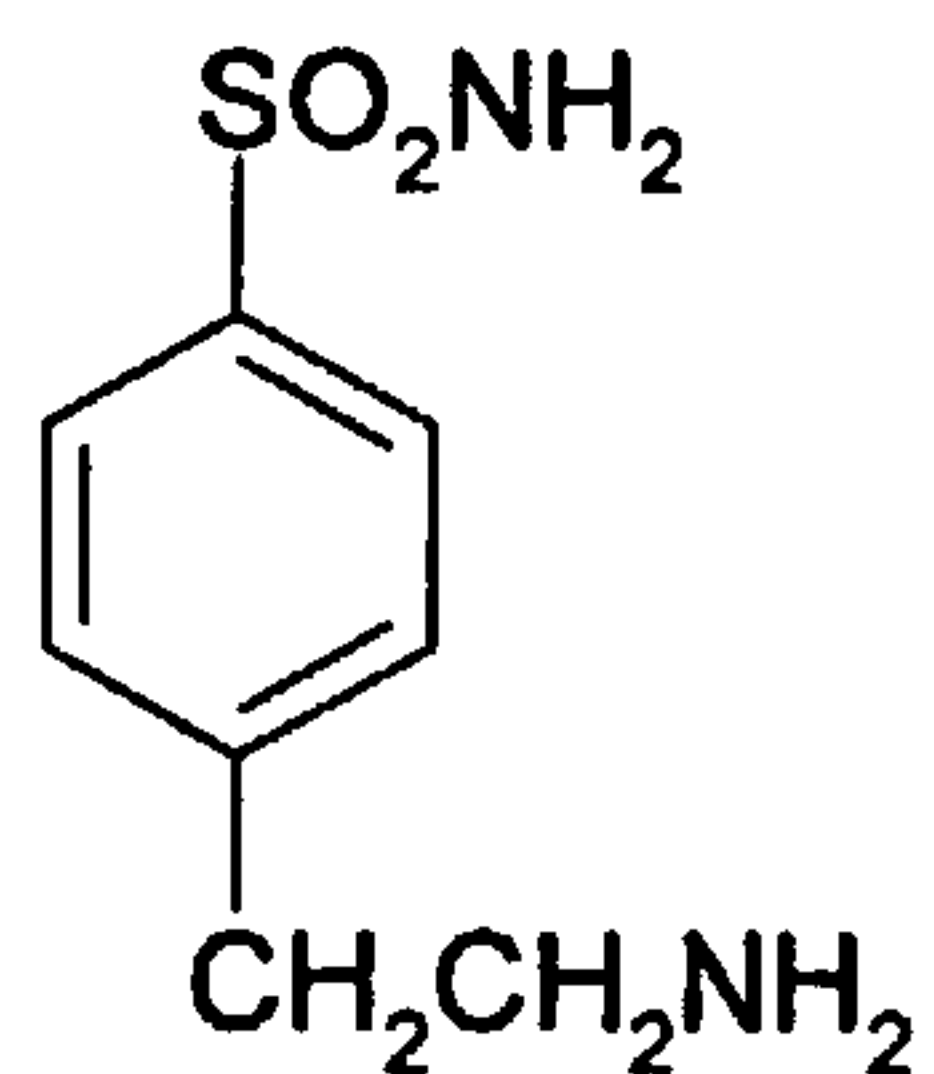
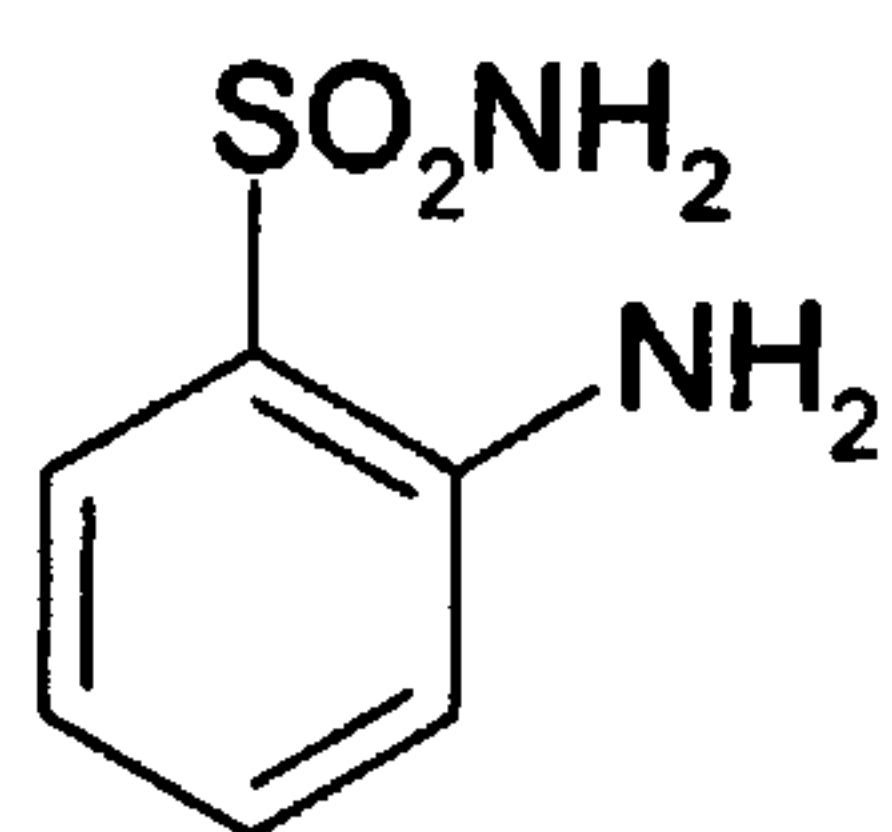
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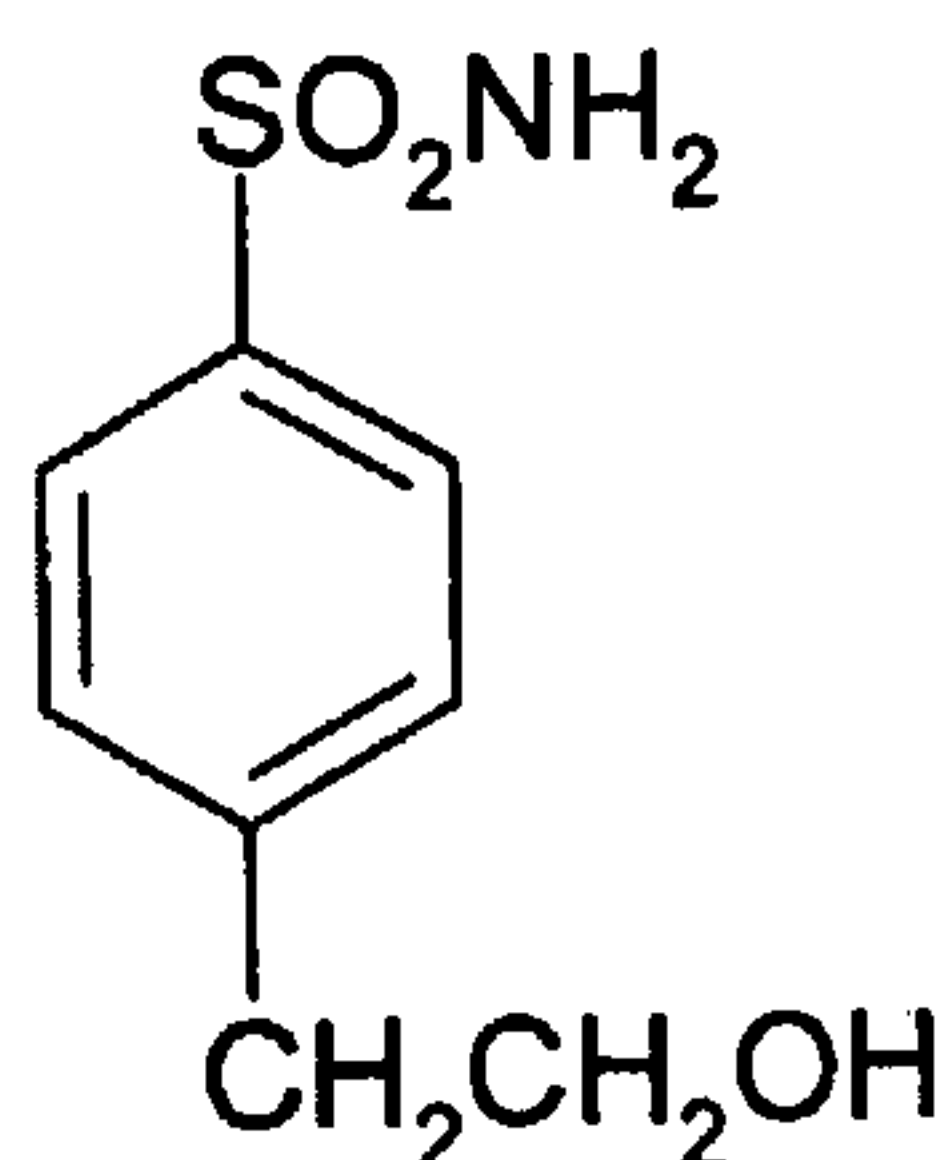
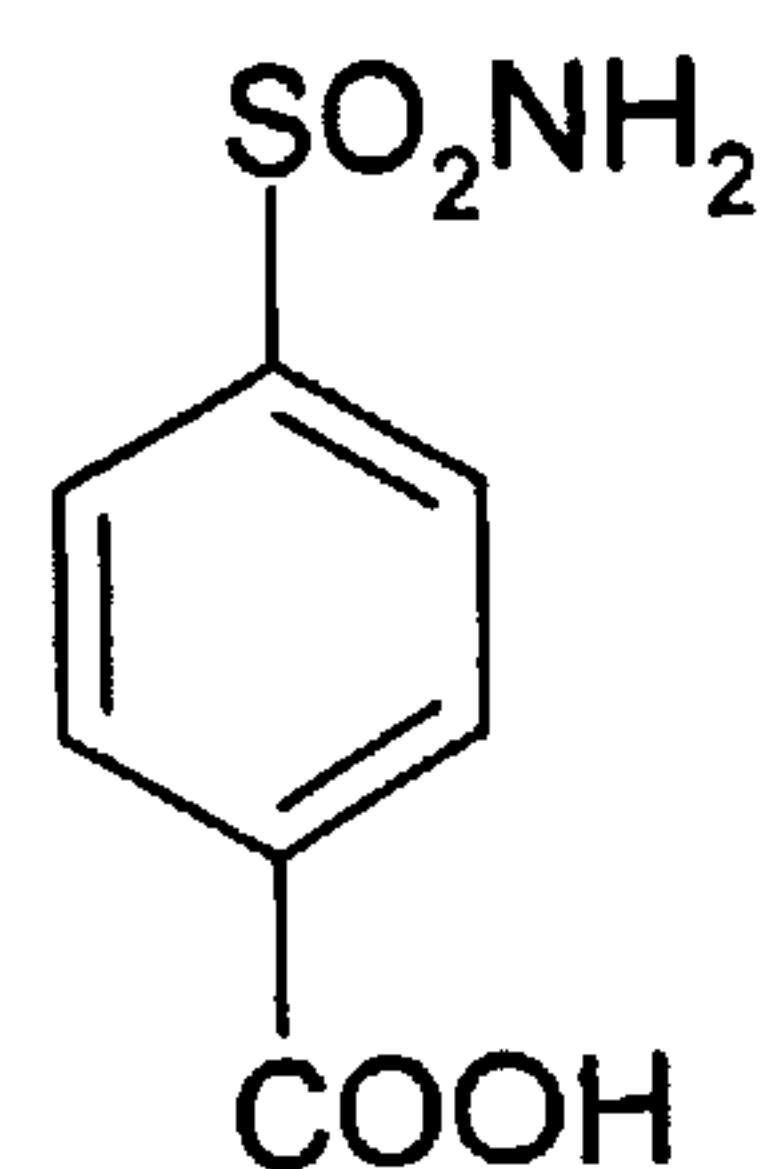


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Exemplary preferred aromatic sulfonamide CA IX-specific inhibitors are selected from the group consisting of:

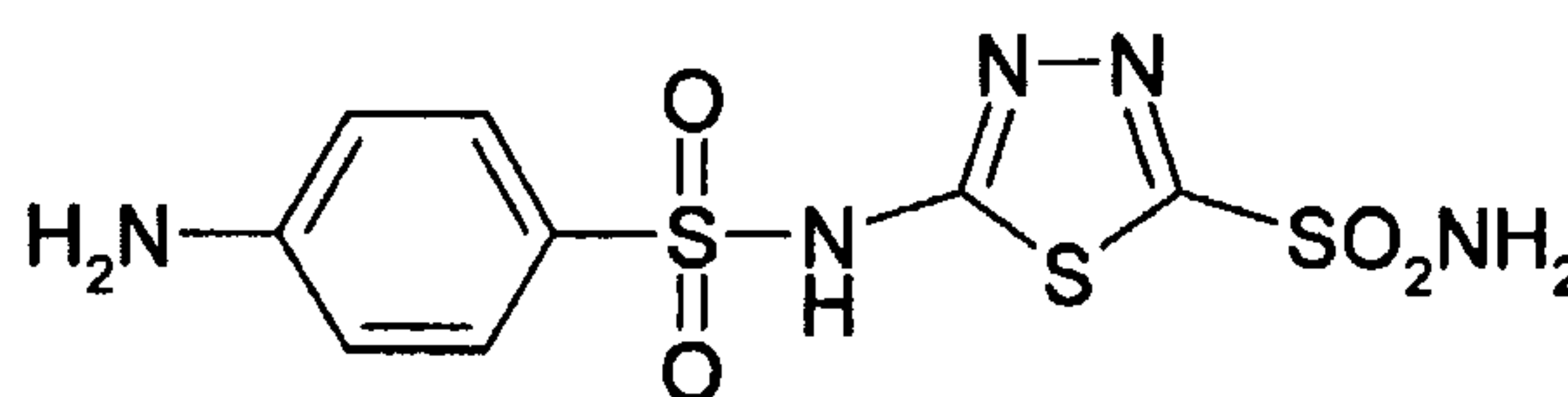


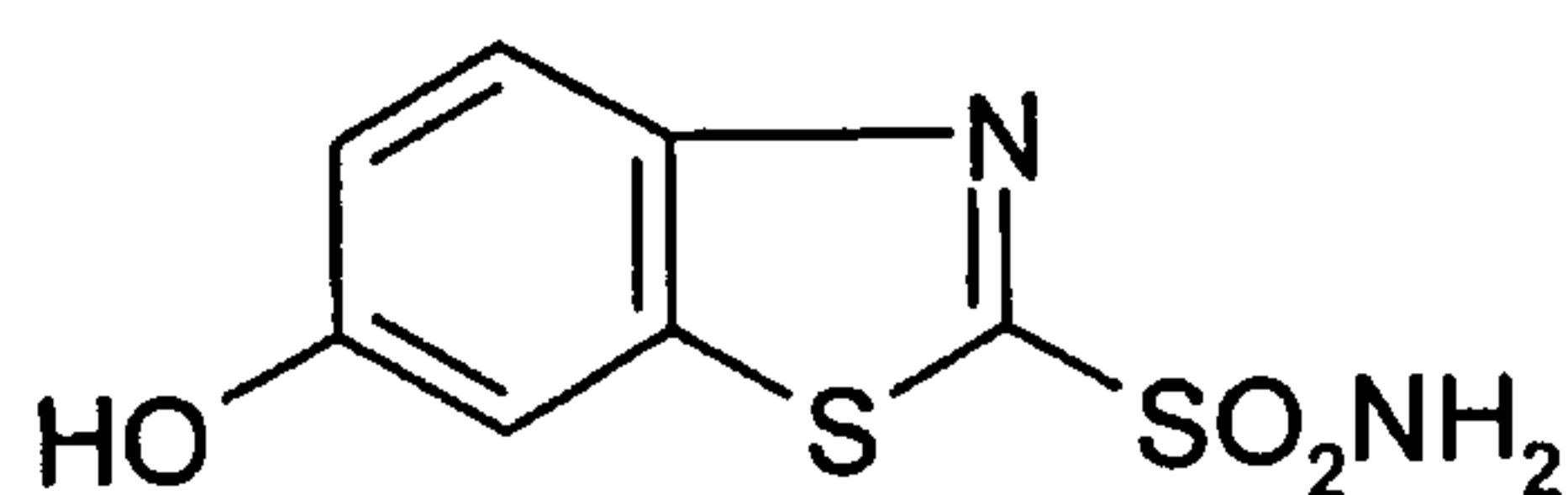
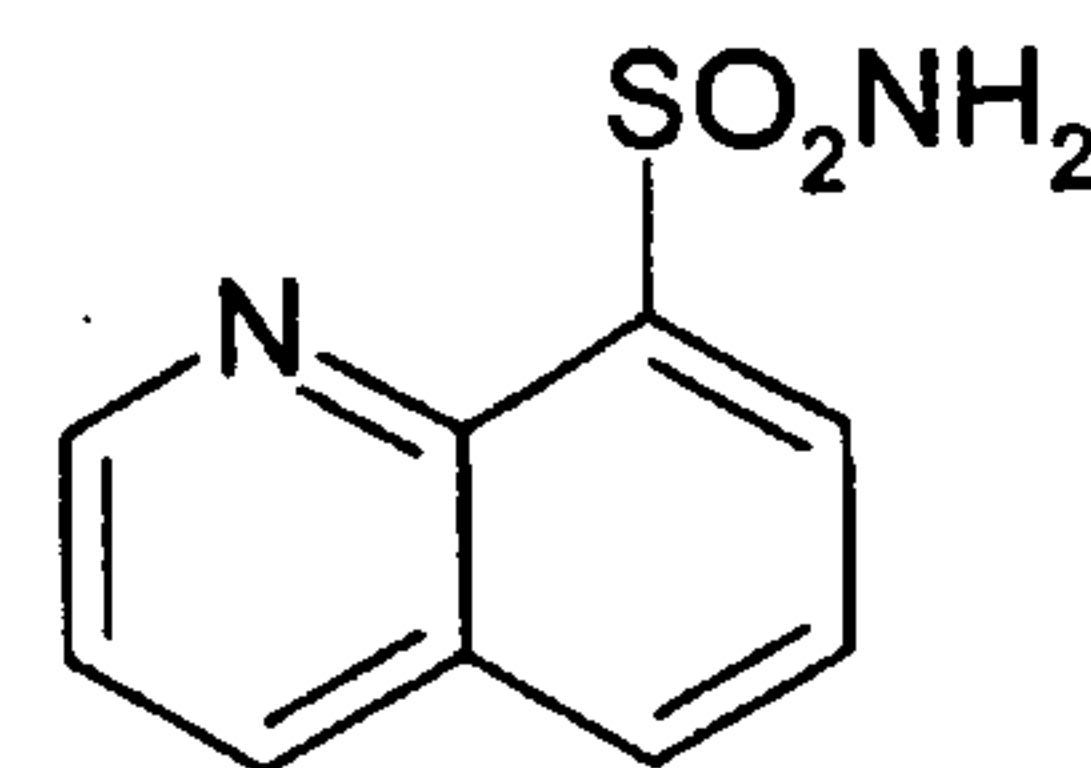
**25****26**

A preferred aromatic sulfonamide CA IX-specific inhibitor can be that wherein a halogen atom is bonded to at least one carbon atom in the aromatic ring of said aromatic sulfonamide.

Preferred heterocyclic sulfonamide CA IX-specific inhibitors can be substituted heterocyclic sulfonamides, wherein said substituted heterocyclic sulfonamide comprises a heterocyclic ring structure bearing a sulfonamide moiety bonded to said ring structure and optionally bearing one or more substituents independently selected from a group consisting of halogeno, nitro, and an alkylamino group, wherein the alkyl radical of said alkylamino group comprises 1 to 4 carbon atoms. Preferred heterocyclic sulfonamide CA IX-specific inhibitors may be halogenated.

Further preferred heterocyclic sulfonamide CA IX-specific inhibitors are selected from the group consisting of:

**14****15**

**21****22**

Further preferred methods of treating mammals for pre-cancerous or cancerous disease, wherein said disease is characterized by overexpression of MN/CA IX protein, comprise administering to said mammal membrane-impermeant CA IX-specific inhibitors. A therapeutically effective amount of such a membrane-impermeant CA IX-specific inhibitor can be administered in a composition comprising the membrane-impermeant compound, wherein said membrane-impermeant inhibitor compound is selected from the group consisting of organic and inorganic molecules, and wherein said membrane-impermeant compound is determined to be a potent inhibitor of MN/CA IX enzymatic activity in a screening assay comprising:

a) preparing serial dilutions of said membrane-impermeant compound and serial dilutions of MN/CA IX protein or a fragment of the MN/CA IX protein that comprises the carbonic anhydrase domain;

b) preincubating a dilution of said membrane-impermeant compound with a dilution of said MN/CA IX protein or said MN/CA IX protein fragment for ten minutes at 20°C;

c) combining said preincubated mixture of said diluted compound and said diluted MN/CA IX protein or protein fragment with a substrate, consisting essentially of a saturated CO₂ solution, phenol red to 0.2mM, Na₂SO₄ to 0.1M, and Hepes buffer (pH 7.5) to 10mM, in a reaction vessel for a period of 10 to 100 seconds at 20°C;

d) concurrently measuring the optical density, at the absorbance maximum of 557 nm, of the contents of said reaction vessel, using a stopped flow spectrophotometer; and

e) determining the inhibition constant K_i of said membrane-impermeant compound,

wherein if said inhibition constant K_i is determined to be less than about 50 nanomolar, said membrane-impermeant compound is determined to be a potent inhibitor of MN/CA IX enzymatic activity. The mammal is preferably a human, and the K_i is preferably less than 35 nM, more preferably less than about 25 nM, and
5 still more preferably less than about 10 nanomolar.

Such a membrane-impermeant CA IX specific inhibitor compound is preferably organic, and more preferably a pyridinium derivative of an aromatic sulfonamide or a pyridinium derivative of a heterocyclic sulfonamide. Such membrane-impermeant CA IX-specific inhibitor compounds are preferably more
10 potent inhibitors of MN/CA IX enzymatic activity than of the enzymatic activity of a carbonic anhydrase selected from the group consisting of CA I, CA II and CA IV, and still more preferably more potent inhibitors of MN/CA IX enzymatic activity than of the enzymatic activity of at least two carbonic anhydrases selected from the group consisting of CA I, CA II and CA IV. Further more preferably, said membrane-
15 impermeant CA IX-specific inhibitor compounds are more potent inhibitors of MN/CA IX enzymatic activity than of the enzymatic activity of each of the carbonic anhydrases in the group consisting of CA I, CA II and CA IV. Since both CA IX and CA IV are membrane bound CAs, it is particularly important that the membrane-impermeant CA IX-specific inhibitor compounds are more potent inhibitors of MN/CA
20 IX enzymatic activity than of the enzymatic activity of CA IV.

A method comprising the following steps provides an exemplary screening assay that can be used to determine the K_i of a compound inhibiting the enzymatic activity of CA IV:

a) preparing serial dilutions of said membrane-impermeant compound
25 and serial dilutions of CA IV;

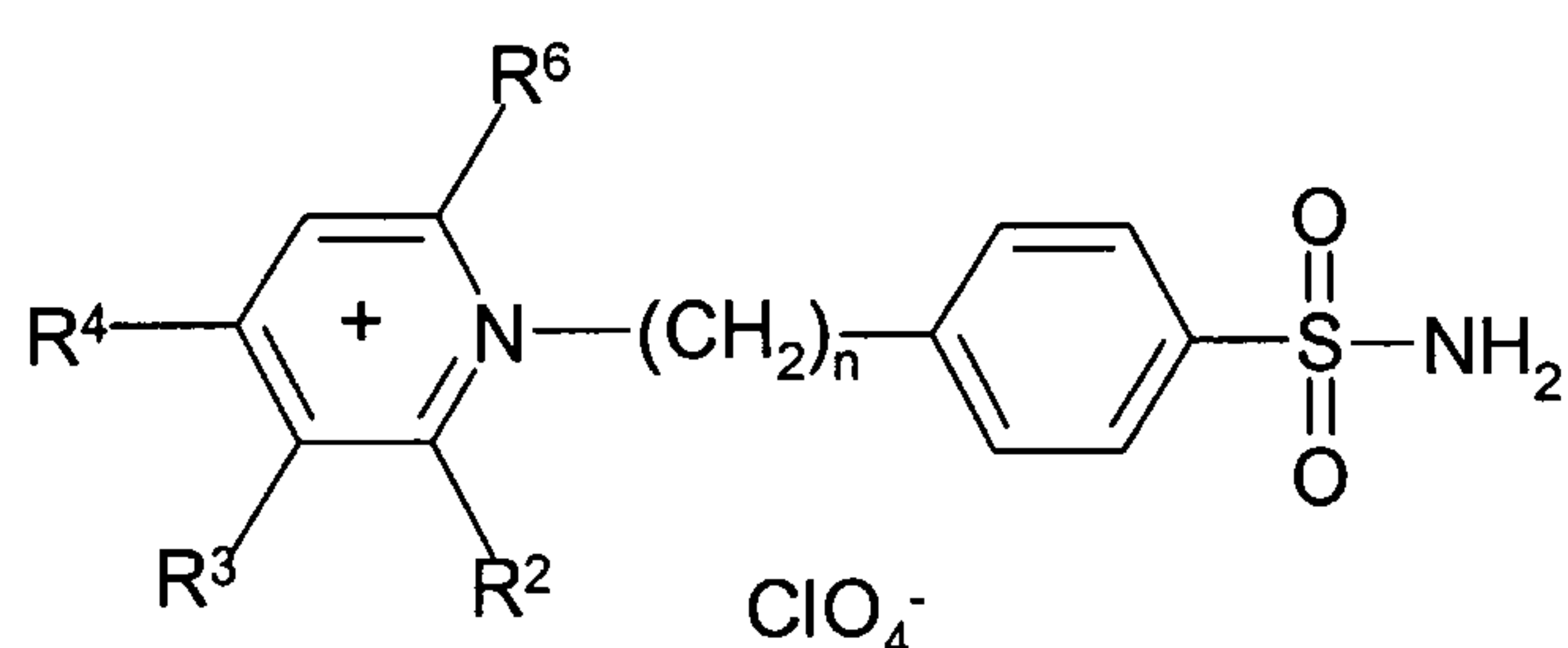
b) preincubating a dilution of said membrane-impermeant compound with a dilution of CA IV for ten minutes at 20°C;

c) combining said preincubated mixture of said compound and said CA IV with a substrate solution, consisting essentially of 4-nitrophenylacetate in
30 anhydrous acetonitrile, in a reaction vessel for a period of 1 to 3 minutes at 25°C;

d) concurrently measuring the optical density, at the absorbance maximum of 400 nm, of the contents of said reaction vessel using a spectrophotometer; and

e) determining the inhibition constant K_i of said membrane-impermeant compound.

Preferred membrane-impermeant CA IX-specific inhibitor compounds that are pyridinium derivatives of aromatic sulfonamides are selected from the group consisting of sulfanilamide, homosulfanilamide and 4-aminoethylbenzenesulfonamide. Preferred pyridinium derivatives of aromatic sulfonamides can have the general formula of:



wherein

n is 0, 1, or 2;

R_2 , R_3 , R_4 and R_6 are each independently selected from the group consisting of hydrogen, alkyl moieties comprising from 1 to 12 carbon atoms, and aryl moieties. Further preferred are such compounds wherein

R_2 is selected from the group consisting of methyl, ethyl, *n*-propyl, *iso*-propyl, *n*-butyl, *tert*-butyl and phenyl;

R_3 is selected from the group consisting of hydrogen and methyl;

R_4 is selected from the group consisting of hydrogen, methyl and phenyl; and

R_6 is selected from the group consisting of methyl, ethyl, *n*-propyl, *iso*-propyl, and phenyl. Still further preferred are such compounds wherein

R_3 is hydrogen;

R_4 and R_6 are phenyl;

when n is 0, R₂ is selected from the group consisting of methyl, ethyl, n-propyl, iso-propyl, n-butyl, and phenyl; and

when n is 1 or 2, R₂ is selected from the group consisting of methyl, ethyl, n-propyl, iso-propyl, n-butyl, tert-butyl, and phenyl. Other

preferred such compounds include those wherein

R₃ is hydrogen;

R₄ is phenyl; and

when n is 0, R₂ and R₆ are the same and are selected from the group consisting of methyl, ethyl, n-propyl, and iso-propyl; and

when n is 1 or 2, R₂ and R₆ are the same and are selected from the group consisting of methyl, ethyl, n-propyl and iso-propyl. Other preferred compounds include those wherein R₂, R₃, R₄ and R₆ are methyl. Still further preferred are such CA IX-specific inhibitor compounds wherein

when n is 0, 1 or 2, R₂, R₄ and R₆ are methyl, and R₃ is hydrogen; or

when n is 1 or 2, R₂ is iso-propyl, R₃ is hydrogen, R₄ is methyl, and R₆ is methyl or iso-propyl; or

when n is 1 or 2, R₂ and R₆ are phenyl, and R₃ and R₄ are hydrogen.

Still more preferred such compounds are those wherein

when n is 2, R₂ and R₆ are methyl, R₃ is hydrogen, and R₄ is phenyl;

or

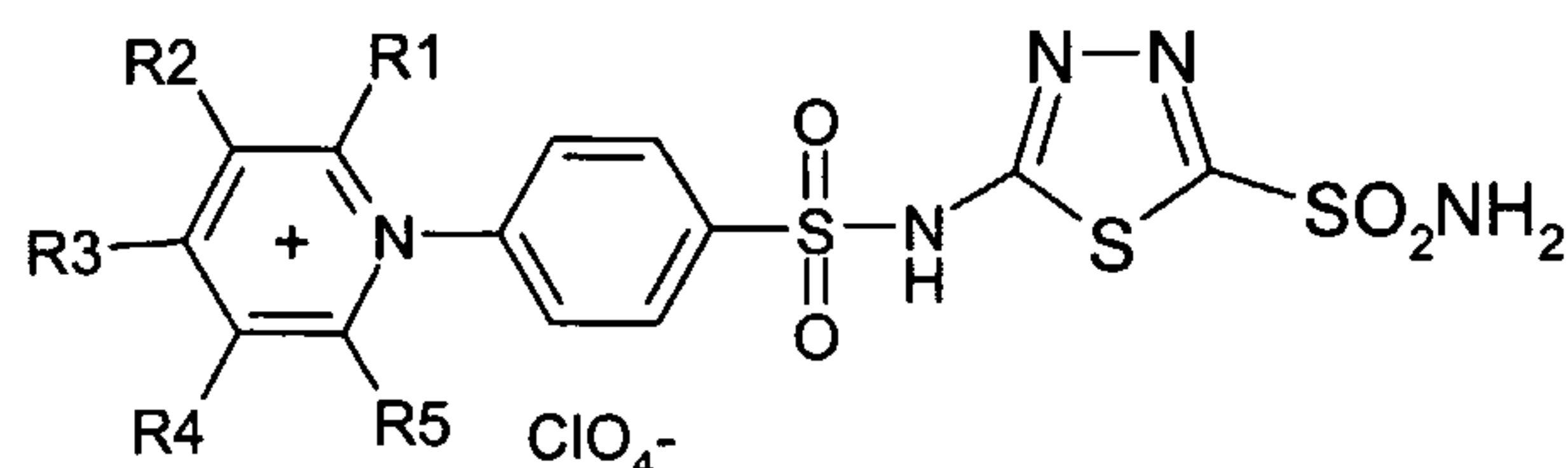
when n is 2, R₂ and R₆ are ethyl, R₃ is hydrogen, and R₄ is phenyl; or

when n is 2, R₂, R₃, R₄ and R₆ are methyl.

When said CA IX-specific inhibitors are membrane-impermeant pyridinium derivatives of a heterocyclic sulfonamides, a preferred compound is a

pyridinium derivative of aminobenzolamide.

Preferred CA IX-specific inhibitor compounds that are pyridinium derivatives of heterocyclic sulfonamides may have the general formula of:



wherein R1, R2, R3, R4 and R5 are each independently selected from the group consisting of hydrogen, alkyl moieties comprising from 1 to 12 carbon atoms, and aryl moieties. Further preferred are such compounds wherein

5 R1 is selected from the group consisting of methyl, ethyl, iso-propyl, n-propyl, n-butyl, *tert*-butyl and phenyl;

R2 is selected from the group consisting of hydrogen and methyl;

R3 is selected from the group consisting of hydrogen, methyl, *n*-nonyl, and phenyl;

R4 is selected from the group consisting of hydrogen and methyl; and

10 R5 is selected from the group consisting of methyl, ethyl, iso-propyl, n-propyl, n-butyl, *tert*-butyl, *n*-nonyl, and phenyl. Further preferred are such compounds wherein

R2 and R4 are hydrogen;

R3 is methyl; and

15 R1 and R5 are the same and selected from the group consisting of methyl, *iso*-propyl, and *tert*-butyl. Still further preferred are such compounds wherein

R2 and R4 are hydrogen;

R3 is phenyl; and

20 R1 and R5 are the same and selected from the group consisting of methyl, ethyl, iso-propyl, n-propyl, n-butyl, and phenyl. Additionally are preferred such compounds wherein

R1 is selected from the group consisting of methyl, ethyl, iso-propyl, n-propyl, and n-butyl;

R2 and R4 are hydrogen; and

25 R3 and R5 are phenyl. Other preferred such compounds are those wherein

R2 and R4 are hydrogen, R3 is hydrogen or methyl, and R1 and R5 are phenyl; or

R1, R2, and R5 are methyl, R3 is phenyl, and R4 is hydrogen; or

30 R1 and R4 are methyl, R2 is hydrogen, and R3 and R5 are *n*-nonyl.

Also preferred such compounds are those wherein

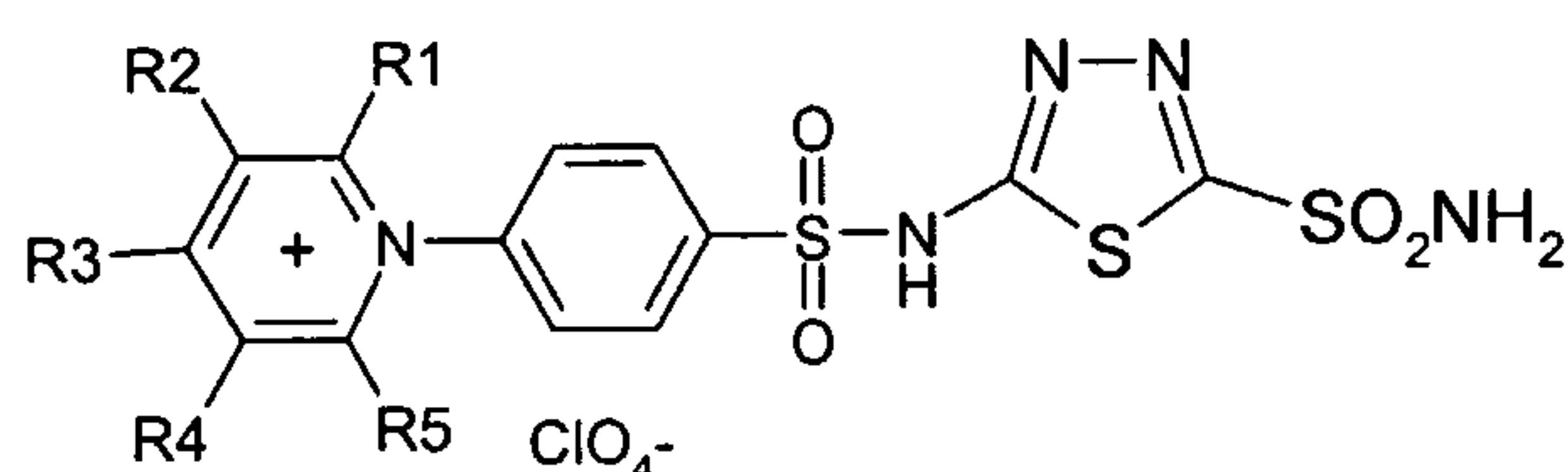
R1 is methyl or iso-propyl, R3 and R5 are methyl, and R2 and R4 are hydrogen; or

R1 and R5 are the same and are methyl or ethyl, R2 and R4 are hydrogen, and R3 is phenyl; or

5 R1, R2, R3 and R5 are methyl, and R4 is hydrogen.

In another aspect, this invention concerns methods of inhibiting tumor growth in a patient having a tumor, the cells of which tumor are characterized by overexpression of MN/CA IX protein, comprising administering to said patient a therapeutically effective amount of a composition comprising a compound, wherein
10 said compound is selected from the group consisting of organic and inorganic molecules, and wherein said compound is determined to be a potent inhibitor of MN/CA IX enzymatic activity in a screening assay as outlined above for MN/CA IX using a saturated CO₂ solution.

Still further, this invention concerns novel compounds that are useful as
15 CA IX-specific inhibitors in a variety of methods disclosed herein. Such novel compounds include pyridinium derivatives of heterocyclic sulfonamides with the general formula of:



20

wherein

R1 is selected from the group consisting of methyl, ethyl, iso-propyl, *n*-propyl, *n*-butyl, *tert*-butyl and phenyl;

R2 is selected from the group consisting of hydrogen and methyl;

25 R3 is selected from the group consisting of hydrogen, methyl, *n*-nonyl and phenyl;

R4 is selected from the group consisting of hydrogen and methyl; and

R5 is selected from the group consisting of methyl, ethyl, iso-propyl, *n*-propyl, *n*-butyl, *tert*-butyl, *n*-nonyl and phenyl, except that

R1 cannot be methyl when R2 and R4 are hydrogen and R3 and R5 are methyl; and

R1 cannot be methyl when R2 and R4 are hydrogen, R3 is phenyl and R5 is methyl; and

5 R1 cannot be phenyl when R2 and R4 are hydrogen and R3 and R5 are phenyl. Preferred such pyridinium derivatives of heterocyclic sulfonamides include those wherein

R2 and R4 are hydrogen;

R3 is methyl; and

10 R1 and R5 are the same and selected from the group consisting of *iso*-propyl and *tert*-butyl, and those wherein

R2 and R4 are hydrogen;

R3 is phenyl; and

15 R1 and R5 are the same and selected from the group consisting of ethyl, *iso*-propyl, *n*-propyl, and *n*-butyl, and further preferably those wherein

R1 is selected from the group consisting of methyl, ethyl, *iso*-propyl, *n*-propyl, *n*-butyl, and *tert*-butyl;

R2 and R4 are hydrogen; and

20 R3 and R5 are phenyl. Still further preferred are those pyridinium derivatives of heterocyclic sulfonamides, wherein

R1 is *iso*-propyl, R3 and R5 are methyl, and R2 and R4 are hydrogen;

or

R2 and R4 are hydrogen, R3 is hydrogen or methyl, and R1 and R5 are phenyl; or

25 R1, R2, and R5 are methyl, R3 is phenyl, and R4 is hydrogen; or

R1, R2, R3 and R5 are methyl and R4 is hydrogen; or

R1 and R4 are methyl, R2 is hydrogen and R3 and R5 are *n*-nonyl.

In another therapeutic aspect of the invention, the CA IX-specific inhibitors can be conjugated to radioisotopes for administration. Also, the CA IX-specific inhibitors can be administered concurrently and/or sequentially with radiation and/or with a therapeutically effective amount in a physiologically acceptable formulation of one or more of the following compounds selected from the group

30

consisting of: conventional anticancer drugs, chemotherapeutic agents, different inhibitors of cancer-related pathways, bioreductive drugs, CA IX-specific antibodies and CA IX-specific antibody fragments that are biologically active. Preferably said CA IX-specific antibodies and/or CA IX-specific antibody fragments are humanized or fully human, and may be attached to a cytotoxic entity.

In another therapeutic aspect, this invention concerns methods of treating a mammal for a precancerous or cancerous disease, wherein said disease is characterized by overexpression of MN/CA IX protein, comprising administering to said mammal a therapeutically effective amount in a physiologically acceptable formulation of a vector conjugated to a potent CA IX-specific inhibitor, wherein said vector expresses a wild-type gene that is absent from or mutated in a CA IX expressing cell, that is precancerous or cancerous, and wherein the wild type gene product has an anticancer effect in said cell; or wherein said vector comprises a gene that expresses a cytotoxic protein. An exemplary wild-type gene would be the von Hippel-Lindau gene known to be directly involved in the constitutive expression of CA IX in renal cell carcinoma.

Preferably said vector comprises a MN/CA IX promoter or a MN/CA IX promoter fragment, wherein said promoter or promoter fragment comprises one or more hypoxia response elements (HREs), and wherein said promoter or promoter fragment is operably linked to said wild-type gene or to said gene that expresses a cytotoxic protein. Preferably the CA IX-specific inhibitor conjugated to the vector has a K_i as determined above in the CO_2 saturation assay to be less than about 50 nM, more preferably less than about 35 nM, still more preferably less than about 25 nM and still further more preferably less than about 10 nM. Preferably, said potent MN/CA IX inhibitor is not selected from the group consisting of acetazolamide, ethoxzolamide, methazolamide and cyanate.

Still in another aspect, this invention concerns methods that are diagnostic or diagnostic and prognostic for precancer or cancer. For example, such methods may comprise contacting a mammalian sample with a CA IX-specific inhibitor conjugated to a label or a visualizing means, and detecting or detecting and quantifying binding of said CA IX-specific inhibitor to cells in said sample by detecting or detecting and quantifying said label or said visualizing means on cells in

said sample, wherein said detection or said detection and quantitation at a level above that for a control sample is indicative of precancerous or cancerous cells that overexpress CA IX in said sample.

Such methods can be of particular diagnostic and prognostic
5 importance by detecting or detecting and quantitating CA IX activated by hypoxic conditions. Hypoxia combined with CA IX overexpression indicates that the mammal from whom the sample was taken is considered to have a poorer prognosis, and decisions on treatment for said mammal are made in view of the presence of said hypoxic conditions. MN/CA IX as a hypoxia marker is useful in general in making
10 therapeutic decisions. For example, a cancer patient whose tumor is known to express MN/CA IX at an abnormally high level would not be a candidate for certain kinds of chemotherapy and radiotherapy, but would be a candidate for hypoxia-selective chemotherapy.

Brown, J.M. [16] points out at page 157 that "solid tumours are
15 considerably less well oxygenated than normal tissues. This leads to resistance to radiotherapy and anticancer chemotherapy, as well as predisposing to increased tumour metastases." Brown explains how tumor hypoxia can be exploited in cancer treatment. One strategy to exploit tumor hypoxia for cancer treatment proposed by Brown [16] is to use drugs that are toxic only under hypoxic conditions. Exemplary
20 and preferred drugs that could be used under that strategy include tirapazamine and AQ4N, a di-N-oxide analogue of mitozantrome.

A second mode of exploiting hypoxia proposed by Brown [16] is by gene therapy strategies developed to take advantage of the selective induction of HIF-1. Brown notes that a tumor-specific delivery system can be developed wherein
25 a promoter that is highly responsive to HIF-1 would drive the expression of a conditionally lethal gene under hypoxic but not normoxic conditions. The MN/CA IX promoter is just such a promoter highly responsive to hypoxia, as well as MN/CA IX promoter fragments comprising one or more HREs. "Expression of an enzyme not normally found in the human body could, under the control of a hypoxia-responsive
30 promoter [the MN/Ca IX promoter], convert a nontoxic pro-drug into a toxic drug in the tumour." [Brown [16], page 160.] Exemplary is the use of the bacterial cytosine

deaminase, which converts the nontoxic 5-fluorocytosine to the anticancer drug 5-fluorouracil (5FU) cited by Brown to Trinh et al. [109].

Ratcliffe et al., U.S. Patent Nos. 5,942,434 and 6,265,390 explain how anti-cancer drugs become activated under hypoxia [119], but that the use of a drug
5 activation system, wherein the enzyme that activates the drug is significantly increased under hypoxia, results in much enhanced therapeutic effect.

This invention further concerns methods for imaging tumors and/or metastases that express CA IX in a patient comprising the administration of a CA IX-specific inhibitor linked to an imaging agent to said patient. A preferred imaging
10 method would encompass scintigraphy.

The assays of this invention are both diagnostic and/or prognostic, i.e., diagnostic/prognostic. The term "diagnostic/ prognostic" is herein defined to encompass the following processes either individually or cumulatively depending upon the clinical context: determining the presence of disease, determining the
15 nature of a disease, distinguishing one disease from another, forecasting as to the probable outcome of a disease state, determining the prospect as to recovery from a disease as indicated by the nature and symptoms of a case, monitoring the disease status of a patient, monitoring a patient for recurrence of disease, and/or determining the preferred therapeutic regimen for a patient. The diagnostic/prognostic methods
20 of this invention are useful, for example, for screening populations for the presence of neoplastic or pre-neoplastic disease, determining the risk of developing neoplastic disease, diagnosing the presence of neoplastic and/or pre-neoplastic disease, monitoring the disease status of patients with neoplastic disease, and/or determining the prognosis for the course of neoplastic disease.

25 The present invention is useful for treating and for screening the presence of a wide variety of preneoplastic/neoplastic diseases including carcinomas, such as, mammary, colorectal, urinary tract, ovarian, uterine, cervical, endometrial, squamous cell and adenosquamous carcinomas; head and neck cancers; mesodermal tumors, such as, neuroblastomas and retinoblastomas; sarcomas, such
30 as osteosarcomas and Ewing's sarcoma; and melanomas. Of particular interest are gynecological cancers including ovarian, uterine, cervical, vaginal, vulval and endometrial cancers, particularly ovarian, uterine cervical and endometrial cancers.

Also of particular interest are cancers of the breast, of gastrointestinal tract, of the stomach including esophagus, of the colon, of the kidney, of the prostate, of the liver, of the urinary tract including bladder, of the lung, and of the head and neck.

Gynecologic cancers of particular interest are carcinomas of the uterine cervix, endometrium and ovaries; more particularly such gynecologic cancers include cervical squamous cell carcinomas, adenosquamous carcinomas, adenocarcinomas as well as gynecologic precancerous conditions, such as metaplastic cervical tissues and condylomas.

The invention provides methods and compositions for evaluating the probability of the presence of malignant or pre-malignant cells, for example, in a group of cells freshly removed from a host. Such an assay can be used to detect tumors, quantitate their growth, and help in the diagnosis and prognosis of disease. The assays can also be used to detect the presence of cancer metastasis, as well as confirm the absence or removal of all tumor tissue following surgery, cancer chemotherapy and/or radiation therapy. It can further be used to monitor cancer chemotherapy and tumor reappearance.

The presence of MN antigen can be detected and/or quantitated using a number of well-defined diagnostic assays. Those in the art can adapt any of the conventional immunoassay formats to detect and/or quantitate MN antigen as herein disclosed. The immunoassays of this invention can be embodied in test kits which comprise the potent CA IX-specific inhibitors of this invention, appropriately labeled and/or linked to a visualizing means, as known in the art. Such test kits can be in solid phase formats, but are not limited thereto, and can also be in liquid phase format, and can be based on immunohistochemical assays, ELISAS, particle assays, radiometric or fluorometric assays either unamplified or amplified, using, for example, avidin/biotin technology, among other assay formats.

Exemplary CA IX-specific inhibitors of the invention are shown herein to treat transfected cells that constitutively express MN/CA IX compared to non-transfected cells with no MN/CA IX expression. The exemplary CA IX-specific inhibitors are shown to inhibit acidification of extracellular pH induced by MN/CA IX in cell cultures exposed to hypoxia.

Further, labeled exemplary CA IX-specific inhibitors, such as labeled sulfonamides, for example, conjugated to fluorescein isothiocyanate (FITC), are shown to bind to the surface of MN/CA IX transfected cells, and not to control cells, only in hypoxia but not in normoxia. Those experiments confirm that CA IX-specific inhibitors, such as the sulfonamide compounds described herein, can specifically target MN/CA IX under conditions characteristic of intratumoral microenvironments.

The CA IX-specific inhibitors of this invention can be used diagnostically and prognostically for precancer and cancer, and to determine the status of a patient, and therapeutically, individually or in different combinations with conventional therapeutic regimens to treat precancers and/or cancer. The CA IX-specific inhibitors may also be used in cancer research.

More particularly for treating precancer and/or cancer, the CA IX-specific inhibitors of this invention can be used to hinder cancer expansion and/or progression by blocking CA IX activity. The CA IX-specific inhibitors can be conjugated to radioisotopes for radiotherapy. The CA IX-specific inhibitors can be combined with CA IX-specific antibodies and a variety of conventional therapeutic drugs, different inhibitors of cancer-related pathways, bioreductive drugs, and/or radiotherapy, wherein different combinations of treatment regimens with the CA IX-specific inhibitors of this invention may increase overall treatment efficacy.

Particularly, the CA IX-specific inhibitors of this invention may be combined with therapy using MN/CA IX-specific antibodies and/or CA IX-specific antibody fragments, preferably humanized CA IX-specific antibodies and/or biologically active fragments thereof, and more preferably fully human CA IX-specific antibodies and/or fully human CA IX-specific biologically active antibody fragments. Said CA IX-specific antibodies and biologically active CA IX-specific antibody fragments, preferably humanized and more preferably fully human, may be conjugated to a cytotoxic entity, for example, a cytotoxic protein, such as ricin A, among many other cytotoxic entities.

Still further, a CA IX-specific inhibitor of this invention could be coupled to a vector for targeted delivery to CA IX-specific expressing cells for gene therapy (for example, with the wild-type von Hippel-Lindau gene), or for effecting the expression of cytotoxic proteins, preferably wherein said vector comprises a MN/CA

IX promoter or MN/CA IX promoter fragment comprising the MN/CA IX hypoxia response element (HRE) or a HRE of another gene, and more preferably wherein the CA IX promoter or CA IX promoter fragment comprises more than one HRE, wherein said HRE or HREs is or are either of MN/CA IX, and/or of other genes
5 and/or of genetically engineered HRE consensus sequences in a preferred context.

Particularly, the CA IX-specific inhibitors of this invention can be used diagnostically/prognostically to detect precancerous and/or cancerous cells by binding to CA IX, preferably to CA IX activated by hypoxic conditions, wherein said CA IX specific inhibitors are coupled to a label or to some visualizing means. Such
10 detection, particularly of hypoxic conditions, and CA IX overexpression, can be helpful in determining effective treatment options, and in predicting treatment outcome and the prognosis of disease development. Further the CA IX-specific inhibitors when labeled or linked to an appropriate visualizing means can be used for imaging tumors and/or metastases that express CA IX.

15 The CA IX-specific inhibitors of this invention can also be used in basic and pre-clinical research. For example, the CA IX-specific inhibitors can be used to study the regulation of CA IX enzyme activity, to study the role of CA IX in tumor growth and metabolism, and to study the role of CA IX in response to treatment by drugs, radiation, inhibitors and other therapeutic regimens.

20 Further methods are disclosed for the preparation of positively-charged, membrane-impermeant heterocyclic sulfonamide CA inhibitors with high affinity for the membrane-bound carbonic anhydrase CA IX. Particularly preferred CA IX-specific inhibitors are pyridinium derivatives of such aromatic and heterocyclic sulfonamides. The general structure of the preferred pyridinium derivatives of
25 sulfonamides can be described as a pyridinium portion attached to the "tail" of an aromatic or heterocyclic sulphonamide portion of the compound.

Further provided are screening assays for compounds that are useful for inhibiting the growth of a vertebrate, preferably mammalian, more preferably human, preneoplastic or neoplastic cell that abnormally expresses MN protein. Said
30 screening assays comprise tests for the inhibition of the enzymatic activity of MN by said compounds. Additional assays provided herein test said compounds for their cell membrane impermeance.

Aspects of the instant invention disclosed herein are described in more detail below.

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Abbreviations

30 The following abbreviations are used herein:

aa	-	amino acid
AAZ	-	acetazolamide
ATCC	-	American Type Culture Collection

	bp	-	base pairs
	BRL	-	Bethesda Research Laboratories
	BRZ	-	brinzolamide
	BSA	-	bovine serum albumin
5	CA	-	carbonic anhydrase
	CAI	-	carbonic anhydrase inhibitor
	CAM	-	cell adhesion molecule
	CARP	-	carbonic anhydrase related protein
	Ci	-	curie
10	cm	-	centimeter
	CNS	-	central nervous system
	cpm	-	counts per minute
	C-terminus	-	carboxyl-terminus
	°C	-	degrees centigrade
15	DCP	-	dichlorophenamide
	DEAE	-	diethylaminoethyl
	DMEM	-	Dulbecco modified Eagle medium
	ds	-	double-stranded
	DZA	-	dorzolamide
20	EDTA	-	ethylenediaminetetraacetate
	EZA	-	ethoxzolamide
	F	-	fibroblasts
	FCS	-	fetal calf serum
	FITC	-	fluorescein isothiocyanate
25	H	-	HeLa cells
	IC	-	intracellular
	kb	-	kilobase
	kbp	-	kilobase pairs
	kd or kDa	-	kilodaltons
30	K _i	-	inhibition constant
	KS	-	keratan sulphate
	LTR	-	long terminal repeat

	M	-	molar
	mA	-	milliampere
	MAb	-	monoclonal antibody
	ME	-	mercaptoethanol
5	MEM	-	minimal essential medium
	min.	-	minute(s)
	mg	-	milligram
	ml	-	milliliter
	mM	-	millimolar
10	MMC	-	mitomycin C
	mmol	-	millimole
	MZA	-	methazolamide
	N	-	normal concentration
	NEG	-	negative
15	ng	-	nanogram
	nm	-	nanometer
	nM	-	nanomolar
	nt	-	nucleotide
	N-terminus	-	amino-terminus
20	ODN	-	oligodeoxynucleotide
	ORF	-	open reading frame
	PA	-	Protein A
	PBS	-	phosphate buffered saline
	PCR	-	polymerase chain reaction
25	PG	-	proteoglycan
	pI	-	isoelectric point
	PMA	-	phorbol 12-myristate 13-acetate
	POS	-	positive
	Py	-	pyrimidine
30	QAS	-	quaternary ammonian sulfonilamide
	QSAR	-	quantitative structure-activity relationship(s)
	RACE	-	rapid amplification of cDNA ends

	RCC	-	renal cell carcinoma
	RIA	-	radioimmunoassay
	RIP	-	radioimmunoprecipitation
	RIPA	-	radioimmunoprecipitation assay
5	RNP	-	RNase protection assay
	RT-PCT	-	reverse transcription polymerase chain reaction
	SAC	-	<u>Staphylococcus aureus</u> cells
	SAR	-	structure-activity relationship
	sc	-	subcutaneous
10	SDS	-	sodium dodecyl sulfate
	SDS-PAGE	-	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
	SINE	-	short interspersed repeated sequence
	SP	-	signal peptide
	SP-RIA	-	solid-phase radioimmunoassay
15	TBE	-	Tris-borate/EDTA electrophoresis buffer
	TC	-	tissue culture
	TCA	-	trichloroacetic acid
	TC media	-	tissue culture media
	TC	-	tissue culture
20	tk	-	thymidine kinase
	TM	-	transmembrane
	Tris	-	tris (hydroxymethyl) aminomethane
	μ Ci	-	microcurie
	μ g	-	microgram
25	μ l	-	microliter
	μ M	-	micromolar

Cell Lines

30	BL21 (DE3)	--	<i>Escherichia coli</i> strain described by Lindskog's group (for CA I, II expression)[Lindskog et al., "Structure-function relations in human carbonic anhydrase II as studied by site-directed
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mutagenesis," in Carbonic anhydrase – From biochemistry and genetics to physiology and clinical medicine, Botre et al., Eds., VCH, Weinheim, pp. 1-13 (1991)]

5 BL21-GOLD -- *Escherichia coli* strain (from Stratagene) used for CA IX
(DE3) expression)

Nucleotide and Amino Acid Sequence Symbols

The following symbols are used to represent nucleotides herein:

10	<u>Base Symbol</u>	<u>Meaning</u>
	A	adenine
	C	cytosine
	G	guanine
15	T	thymine
	U	uracil
	I	inosine
	M	A or C
	R	A or G
20	W	A or T/U
	S	C or G
	Y	C or T/U
	K	G or T/U
	V	A or C or G
25	H	A or C or T/U
	D	A or G or T/U
	B	C or G or T/U
	N/X	A or C or G or T/U

30 There are twenty main amino acids, each of which is specified by a different arrangement of three adjacent nucleotides (triplet code or codon), and which are linked together in a specific order to form a characteristic protein. A three-

letter or one-letter convention is used herein to identify said amino acids, as, for example, in Figure 1 as follows:

	<u>Amino acid name</u>	<u>3 Ltr. Abbrev.</u>	<u>1 Ltr. Abbrev.</u>
5	Alanine	Ala	A
	Arginine	Arg	R
	Asparagine	Asn	N
10	Aspartic Acid	Asp	D
	Cysteine	Cys	C
	Glutamic Acid	Glu	E
	Glutamine	Gln	Q
	Glycine	Gly	G
15	Histidine	His	H
	Isoleucine	Ile	I
	Leucine	Leu	L
	Lysine	Lys	K
	Methionine	Met	M
20	Phenylalanine	Phe	F
	Proline	Pro	P
	Serine	Ser	S
	Threonine	Thr	T
	Tryptophan	Trp	W
25	Tyrosine	Tyr	Y
	Valine	Val	V
	Unknown or other		X

BRIEF DESCRIPTION OF THE FIGURES

30 Figure 1A-C provides the nucleotide sequence for MN/CA IX full-length cDNA [SEQ ID NO: 1]. Figure 1 A-C also sets forth the predicted amino acid sequence [SEQ ID NO: 2] encoded by the cDNA.

Figure 2A-F provides a 10,898 bp complete genomic sequence of MN/CA9 [SEQ ID NO: 3]. The base count is as follows: 2654 A; 2739 C; 2645 G; and 2859 T. The 11 exons are in general shown in capital letters, but exon 1 is considered to begin at position 3507 as determined by RNase protection assay.

5 Figure 3 provides an exon-intron map of the human MN/CA9 gene. The positions and sizes of the exons (numbered, cross-hatched boxes), Alu repeat elements (open boxes) and an LTR-related sequence (first unnumbered stippled box) are adjusted to the indicated scale. The exons corresponding to individual MN/CA IX protein domains are enclosed in dashed frames designated PG (proteoglycan-like domain), CA (carbonic anhydrase domain), TM (transmembrane anchor) and IC (intracytoplasmic tail). Below the map, the alignment of amino acid sequences illustrates the extent of homology between the MN/CA IX protein PG region (aa 53-111) [SEQ ID NO: 4] and the human aggrecan (aa 781-839) [SEQ ID NO: 5].

15 Figure 4 A-B shows the chemical structures of the 26 different sulfonamide compounds tested in Example 1.

Figure 5 shows the scheme for the general synthesis of compounds 71 – 91 of Example 3 (**Scheme 1**).

Figure 6 shows the scheme for the reaction between a pyrylium salt and an amine (**Scheme 2**), as described in Example 3.

DETAILED DESCRIPTION

20 The novel methods of the present invention comprise inhibiting the growth of tumor cells which overexpress MN protein with compounds that inhibit the enzymatic activity of MN protein. Said compounds are organic or inorganic, preferably organic, more preferably sulfonamides. Still more preferably, said compounds are pyridinium derivatives of aromatic or heterocyclic sulfonamides. These preferred pyridinium derivatives of sulfonamides are likely to have fewer side effects than other compounds in three respects: they are small molecules, they are membrane-impermeant, and they are specific potent inhibitors of the enzymatic activity of the tumor-associated MN/CA IX protein.

30 The use of oncoproteins as targets for developing new cancer therapeutics is considered conventional by those of skill in the art. [See, e.g.,

Mendelsohn and Lippman [61]. However, the application of such approaches to MN is new. In comparison to other tumor-related molecules (e.g. growth factors and their receptors), MN has the unique property of being differentially expressed in preneoplastic/neoplastic and normal tissues, which are separated by an anatomic
5 barrier.

The pyridinium derivatives of sulfonamides of the present invention can be formed, for example, by creating bonds between pyrylium salts and aromatic or heterocyclic sulfonamide reagents, as described below. The aromatic or heterocyclic sulfonamide portion of a pyridinium salt of a sulfonamide compound can be called
10 the "head," and the pyridinium portion can be called the "tail."

It can be appreciated by those of skill in the art that various other types of linkages can couple the pyridinium portion with the sulfonamide portion. It can further be appreciated that alternate methods, in addition to those disclosed herein, can be used to make the pyridinium derivatives of the present invention.

15 As used herein, "cancerous" and "neoplastic" have equivalent meanings, and "precancerous" and "preneoplastic" have equivalent meanings.

As used herein, the term "aromatic" when applied to sulphonamide structures means "comprising an aromatic ring, without an additional heterocyclic ring." The term "heterocyclic" when applied to sulphonamide structures means
20 "comprising a heterocyclic ring, with or without an additional aromatic ring."

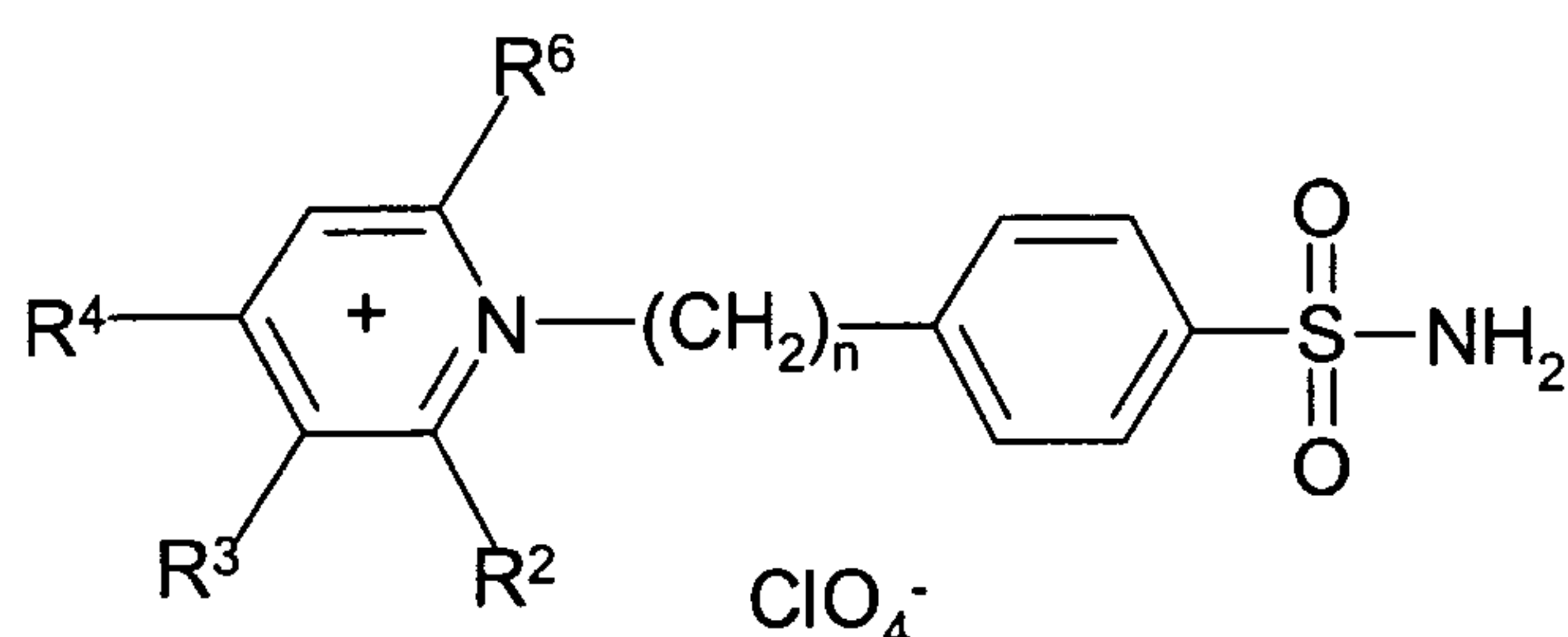
As used herein, the term "alkyl", alone or in combination, refers to a straight-chain or branched-chain alkyl radical containing from 1 to 12, preferably from 1 to 6 and more preferably from 1 to 4, carbon atoms. Examples of such radicals include, but are not limited to, methyl, ethyl, n-propyl, isopropyl, n-butyl, isobutyl,
25 sec-butyl, tert-butyl, pentyl, iso-amyl, hexyl, decyl and the like.

The term "aryl", alone or in combination, means a phenyl or naphthyl radical which optionally carries one or more substituents selected from alkyl, alkoxy, halogen, hydroxy, amino, nitro, cyano, haloalkyl, carboxy, alkoxycarbonyl, cycloalkyl, heterocycloalkyl, amido, mono and dialkyl substituted amino, mono and dialkyl
30 substituted amido and the like, such as phenyl, p-tolyl, 4-methoxyphenyl, 4-(tert-butoxy)phenyl, 3-methyl-4-methoxyphenyl, 4-fluorophenyl, 4-chlorophenyl, 3-nitrophenyl, 3-aminophenyl, 3-acetamidophenyl, 4-acetamidophenyl, 2-methyl-3-

acetamidophenyl, 2-methyl-3-aminophenyl, 3-methyl-4-aminophenyl, 2-amino-3-methylphenyl, 2,4-dimethyl-3-aminophenyl, 4-hydroxyphenyl, 3-methyl-4-hydroxyphenyl, 1-naphthyl, 2-naphthyl, 3-amino-1-naphthyl, 2-methyl-3-amino-1-naphthyl, 6-amino-2-naphthyl, 4,6-dimethoxy-2-naphthyl and the like.

5 Preferred sulfonamides of the present invention are aromatic and heterocyclic sulfonamides. The structures of representative sulfonamides of this group, designated **1-26**, are shown in Figure 4.

More preferred sulfonamides of the present invention are pyridinium derivatives of aromatic sulfonamides and have the general formula (**A**) below,



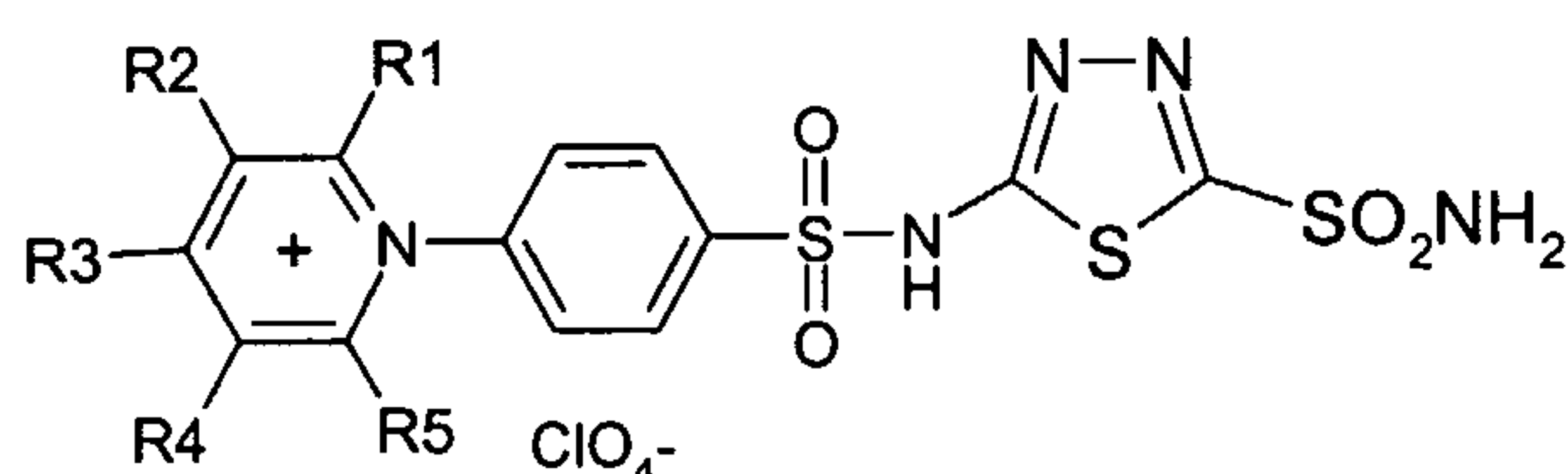
A

wherein n is 0, 1, or 2; and R2, R3, R4 and R6 are each independently selected from the group consisting of hydrogen, alkyls and aryls. The structures of representative sulfonamides of this group, designated **27** through **70**, are shown as derivatives of the general structure (**A**), in Table 2.

15

Alternatively, more preferred sulfonamides of the present invention are pyridinium derivatives of heterocyclic sulfonamides and have the general formula (**B**) below, wherein said pyridinium derivative of a heterocyclic sulfonamide has the general formula of:

20



B

wherein R1, R2, R3, R4 and R5 are each independently selected from the group consisting of hydrogen, alkyls and aryls. The structures of representative sulfonamides of this group, designated **71** through **91**, are shown as derivatives of the general structure (**B**), in Table 3.

5 Representative sulfonamide derivatives of the group of compounds represented by the general formulas (**A**) and (**B**) have CA IX inhibitory activity, and are potentially useful therapeutically as anticancer agents in treating MN-associated tumors.

10 Further, biologic activity of the identified sulfonamides will be tested in vitro by inhibition of the carbonic anhydrase enzymatic activity of the MN protein, by effects on cell morphology and growth characteristics of MN-related tumor cells (HeLa) and of control cells [104]. In vivo screening will be carried out in nude mice that have been injected with HeLa cells.

15 Representative Sulfonamide Inhibitors of CA IX

The sulfonamides investigated in Example 1 for the inhibition of the tumor-associated isozyme CA IX, of types **1-26** are shown in Figure 4A-B. Compounds **1-6**, **11-12**, **20** and **26** are commercially available, whereas **7-10** [43], **13-19** [24, 90, 97] and **21-25** [79] were prepared as reported earlier. The six clinically
20 used compounds were also assayed. For Example 2 compounds (pyridinium derivatives of aromatic sulfonamides), reaction of sulfanilamide, homosulfanilamide or 4-(2-aminoethyl)-benzenesulfonamide with 2,6-di-, 2,4,6-tri- or 2,3,4,6-tetrasubstituted pyrylium salts afforded the pyridinium salts **27-70** investigated here, by the general Bayer - Piccard synthesis [9, 10, 97].

25 As described in Example 3, a series of positively-charged sulfonamides, designated here as compounds **71-91**, were obtained by reaction of aminobenzolamide (5-(4-aminobenzenesulfonylamino)-1,3,4-thiadiazole-2-sulfonamide) with tri-/tetra-substituted pyrylium salts possessing alkyl-, aryl- or combinations of alkyl and aryl groups at the pyridinium ring (described below).
30 Three of these compounds (**71**, **75**, and **87**) have been described elsewhere [25, 85]; all other compounds of this series are new.

Heterocyclic Sulfonamide Inhibitors of CA IX:
Synthesis of Pyridinium Derivatives of Aminobenzolamide

Chemistry: Reaction of aminobenzolamide (5-(4-

5 aminobenzenesulfonylamino)-1,3,4-thiadiazole-2-sulfonamide) [97] with 2,6-di-,
2,4,6-tri- or 2,3,4,6-tetrasubstituted pyrylium salts afforded the pyridinium salts **71-91**
investigated here, by the general synthesis of such derivatives with nucleophiles
(Scheme 1 as shown in Figure 5) [6, 26, 108].

Preparation of compounds: A large number of positively-charged
10 sulfonamides, prepared by reaction of amino-sulfonamides with pyrylium salts [23,
88, 89] were recently reported by this group, and generally tested as inhibitors of the
"classical" isozymes CA I, II and IV [81, 96, 97, 98]. Based on QSAR studies on
several series of CA inhibitors, including some positively-charged derivatives [23, 88,
89], it emerged that the enhancement of CA inhibitory activity is correlated with
15 increased positive charges on the heterocyclic/aromatic ring incorporated in such
molecules, as well as with "long" inhibitor molecules *per se* (i.e., molecules extending
on the direction passing through the Zn(II) ion of the enzyme, the sulfonamide
nitrogen atom and the long axis of the inhibitor) [23, 88, 89]. It appeared thus of
interest to try to explore this result, designing positively-charged, long sulfonamide
20 CAIs. Thus, we thought of attaching substituted-pyridinium moieties to an already
potent and long-molecule CAI suitable for reaction with pyrylium salts, i.e.,
aminobenzolamide [97]. Indeed, this compound acts as a very potent CAI against
isozymes I, II and IV (with inhibition constants in the low nanomolar range – see later
in the text). The substitution pattern of the pyridinium ring was previously shown [81,
25 96, 97, 98] to be critical for the biological activity of this type of sulfonamide CAIs.
Thus, a large series of of 2,4,6-trialkylpyridinium-; 2,6-dialkyl-4-phenylpyridinium-; 2-
alkyl-4,6-diphenylpyridinium-; 2,4,6-triphenylpyridinium-, together with various 2,6-
disubstituted-pyridinium and 2,3,5,6-tetrasubstituted-pyridinium aminobenzolamide
derivatives have been prepared by the reaction described in Scheme 1 (Shown in
30 Figure 5).

Although apparently simple, the reaction between a pyrylium salt and
an amine, leading to pyridinium salts, is in reality a complicated process (Scheme 2,
shown in Figure 6), as established by detailed spectroscopic and kinetic data from

shown in Figure 6), as established by detailed spectroscopic and kinetic data from Balaban's and Katritzky's groups [6, 26, 108]. 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 **IV** (depicted in Figure 6), which by deprotonation
5 in the presence of bases lead to the 2-amino-tetrahydropyran derivatives **V**. 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 [6, 26, 108].
10 The derivatives **V** are generally unstable, being tautomers with the ketodieneamines **VI** which are the key intermediates for the conversion of pyryliums into pyridiniums [6, 26, 108]. In acidic media, in the rate-determining step of the whole process, ketodieneamines **VI** may be converted to the corresponding pyridinium salts **VII**, although other products, such as vinylogous amides with diverse structures have
15 also been isolated in such reactions [6, 26, 108]. A supplementary complication appears when the moiety substituting the 2- and/or 6-position(s) of the pyrylium ring is methyl, cases in which a concurrent cyclisation with formation of the anilines **VIII** in addition to the pyridinium salts **VII**, may take place too [6, 26, 108]. These concurrent reactions mentioned above are generally important when the amine to be
20 converted into the pyridinium salt possesses weak nucleophilicity or basicity. This happens to be the case of aminobenzolamide. In fact, reaction of aminobenzolamide 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
25 150 °C; reaction times between 15 min and 48 hours, etc) led only to the isolation of the unreacted raw materials. The only conditions which led to the formation of the pyridinium salts **III** (depicted in Figure 5) were the following: anhydrous methanol in the presence of acetic anhydride as solvent and triethylamine as catalysts for the deprotonation of the intermediates **IV**. Acetic anhydride had the role of reacting with
30 the water formed in the condensation reaction. This water may in fact act as a competitive nucleophile with aminobenzolamide when reacting with the pyrylium cation, and as a consequence the yields in pyridinium salts would dramatically be

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 refluxation in the presence of acetic acid (2-5 hours). Still the yields were not always good, especially for the 2-methyl-containing derivatives.

5

Preparation of MN Proteins and/or Polypeptides

The terms "MN/CA IX" and "MN/CA9" are herein considered to be synonyms for MN. Also, the G250 antigen is considered to refer to MN protein/polypeptide [112].

10

Zavada et al., WO 93/18152 and/or WO 95/34650 disclose the MN cDNA sequence shown herein in Figure 1A-1C [SEQ ID NO: 1], the MN amino acid sequence [SEQ ID NO: 2] also shown in Figure 1A-1C, and the MN genomic sequence [SEQ ID NO: 3] shown herein in Figure 2A-2F. The MN gene is organized into 11 exons and 10 introns.

15

The first thirty seven amino acids of the MN protein shown in Figure 1A-1C is the putative MN signal peptide [SEQ ID NO: 6]. The MN protein has an extracellular domain [amino acids (aa) 38-414 of Figure 1A-1C [SEQ ID NO: 7], a transmembrane domain [aa 415-434; SEQ ID NO: 8] and an intracellular domain [aa 435-459; SEQ ID NO: 9]. The extracellular domain contains the proteoglycan-like domain [aa 53-111; SEQ ID NO: 4] and the carbonic anhydrase (CA) domain [aa 135-391; SEQ ID NO: 5].

20

25

The phrase "MN proteins and/or polypeptides" (MN proteins/polypeptides) is herein defined to mean proteins and/or polypeptides encoded by an MN gene or fragments thereof. An exemplary and preferred MN protein according to this invention has the deduced amino acid sequence shown in Figure 1. Preferred MN proteins/polypeptides are those proteins and/or polypeptides that have substantial homology with the MN protein shown in Figure 1. For example, such substantially homologous MN proteins/polypeptides are those that are reactive with the MN-specific antibodies, preferably the Mab M75 or its equivalent. The VU-M75 hybridoma that secretes the M75 Mab was deposited at the ATCC under HB 11128 on September 17, 1992.

30

A "polypeptide" or "peptide" is a chain of amino acids covalently bound by peptide linkages and is herein considered to be composed of 50 or less amino acids. A "protein" is herein defined to be a polypeptide composed of more than 50 amino acids. The term polypeptide encompasses the terms peptide and

5 oligopeptide.

It can be appreciated that a protein or polypeptide produced by a neoplastic cell in vivo could be altered in sequence from that produced by a tumor cell in cell culture or by a transformed cell. Thus, MN proteins and/or polypeptides which have varying amino acid sequences including without limitation, amino acid
10 substitutions, extensions, deletions, truncations and combinations thereof, fall within the scope of this invention. It can also be appreciated that a protein extant within body fluids is subject to degradative processes, such as, proteolytic processes; thus, MN proteins that are significantly truncated and MN polypeptides may be found in body fluids, such as, sera. The phrase "MN antigen" is used herein to encompass
15 MN proteins and/or polypeptides.

It will further be appreciated that the amino acid sequence of MN proteins and polypeptides can be modified by genetic techniques. One or more amino acids can be deleted or substituted. Such amino acid changes may not cause any measurable change in the biological activity of the protein or polypeptide and
20 result in proteins or polypeptides which are within the scope of this invention, as well as, MN muteins.

The MN proteins and polypeptides of this invention can be prepared in a variety of ways according to this invention, for example, recombinantly, synthetically or otherwise biologically, that is, by cleaving longer proteins and
25 polypeptides enzymatically and/or chemically. A preferred method to prepare MN proteins is by a recombinant means. Particularly preferred methods of recombinantly producing MN proteins are described below. A representative method to prepare the MN proteins shown in Figure 1 or fragments thereof would be to insert the full-length or an appropriate fragment of MN cDNA into an appropriate
30 expression vector as exemplified in the Materials and Methods section.

MN Gene

Figure 1A-C provides the nucleotide sequence for a full-length MN cDNA clone [SEQ ID NO: 1] isolated as described in Zavada et al., WO 95/34650. Figure 2A-F provides a complete MN genomic sequence [SEQ ID NO: 3].

5 The ORF of the MN cDNA shown in Figure 1 has the coding capacity for a 459 amino acid protein with a calculated molecular weight of 49.7 kd. The overall amino acid composition of the MN/CA IX protein is rather acidic, and predicted to have a pI of 4.3. Analysis of native MN/CA IX protein from CGL3 cells by two-dimensional electrophoresis followed by immunoblotting has shown that in
10 agreement with computer prediction, the MN/CA IX is an acidic protein existing in several isoelectric forms with pIs ranging from 4.7 to 6.3.

The CA domain is essential for induction of anchorage independence, whereas the TM anchor and IC tail are dispensable for that biological effect. The MN protein is also capable of causing plasma membrane ruffling in the transfected cells
15 and appears to participate in their attachment to the solid support. The data evince the involvement of MN in the regulation of cell proliferation, adhesion and intercellular communication.

Enzymatic Screening Assays

20 Assays are provided herein for the screening of compounds for inhibition of the enzymatic activity of the MN protein. Such assays comprise the incubation of said compound with said MN protein and with a substrate selected from the group consisting of saturated CO₂ and 4-nitrophenylacetate, preferably saturated CO₂, and determination of the inhibition constant K_i of said compound, wherein said
25 enzymatic activity of the MN protein is measured by the pH change of an indicator by stopped flow spectrophotometer.

Screening of representative heterocyclic and aromatic sulfonamides for inhibition of MN protein: From Example 1, it was found that the inhibition profile of isozyme CA IX is very different from that of the classical isozymes CA I and II
30 (cytosolic) as well as CA IV (membrane-bound). The following particular features may be noted: (i) all the 32 sulfonamides investigated in Example 1 act as CA IX inhibitors, with inhibition constants in the range of 14-285 nM (the corresponding

affinities for the other three isozymes vary in a much wider range, as seen from data of Table 1). Based on these data, it can be noted that CA IX is a sulfonamide avid CA, similarly to CA II, the isozyme considered up to now to be responsible for the majority of pharmacological effect of sulfonamides [22, 29, 83,93, 94, 95, 102]. Still, many other differences are observed between CA IX and other isozymes for which inhibitors were developed for clinical use; (ii) for CA I, II and IV, generally, aromatic sulfonamides behave as weaker inhibitors as compared to heterocyclic derivatives (compare **1-6**, or **DCP**), as aromatic compounds, with **15**, **21**, **AAZ**, **MZA**, **EZA**, **DZA** or **BRZ** among others (as heterocyclic sulfonamides). In the case of CA IX, such a fine distinction is rather difficult to be made, since both aromatic (such as **1**, **6**, **11**, **12**, **17**, **18**, **22-26**) derivatives, as well as heterocyclic compounds (such as **14**, **15**, **21**, and the clinically used sulfonamides – except dichlorophenamide) possess rather similar inhibition constants, in the range of 14-50 nM; (iii) orthanilamide derivatives (such as **1**, **17** and **22**) behave as very potent CA IX inhibitors (K_i -s in the range of 20-33 nM), although they are weak or medium-weak inhibitors of CA I, II and IV; (iv) 1,3-benzene-disulfonamide derivatives (such as **11**, **12** and **DCP**) are again strong CA IX inhibitors, with K_i -s in the range of 24-50 nM, although their CA II, I and IV inhibition profile is not particularly strong; (v) metanilamide **2**, sulfanilamide **3**, and 4-hydrazino-benzenesulfonamide **4** show CA IX inhibition data quite similar with those against CA II, whereas homosulfanilamide **5** and 4-aminoethyl-benzensulfonamide **6** act as better CA IX inhibitors as compared to CA II inhibition; (vi) the halogenosulfanilamides **7-10** are much weaker inhibitors of CA IX than of CA II, a finding difficult to interpret at this moment; (vii) the strongest CA II inhibitor among the investigated compounds, 4-aminobenzolamide **15** (K_i of 2 nM) is not the strongest CA IX inhibitor (K_i of 38 nM). Instead, the best CA IX inhibitor detected so far is the ethoxzolamide phenol **21** (K_i of 14 nM). It is interesting to note that **21** and **EZA** have the same affinity for CA II, whereas their affinity for CA IX is rather different, with the phenol more active than the ethoxy-derivative; (viii) among the clinically used compounds, the best inhibitor is acetazolamide, followed by methazolamide, ethoxzolamide and brinzolamide. The most ineffective (but appreciably inhibiting the isozyme IX) are dichlorophenamide and dorzolamide; (ix) sulfonamides **20** and **22-26** behave as very good CA IX inhibitors, with K_i -s in the

range of 16-32 nM, being slightly more effective than the clinically used CAIs mentioned above, and among the best CA IX inhibitors detected so far. It is thus envisageable that such compounds may be used as lead molecules for obtaining more potent and eventually specific CA IX inhibitors, with applications as antitumor agents.

Screening of representative pyridinium derivatives of aromatic sulfonamides for inhibition of MN protein: From Example 2, wherein membrane-impermeant pyridinium derivatives of sulfonamides were tested for their ability to inhibit the enzymatic activity of CA IX, the following conclusions were drawn from data of Table 2: (i) for a given substitution pattern of the pyridinium ring, the 4-aminoethyl-benzenesulfonamide derivatives **55-70** were more active than the corresponding homosulfanilamide derivatives **39-54**, which in turn were more active than the corresponding sulfanilamides **27-38**. This behavior has also been observed for the other three investigated isozymes [96]; (ii) some of the derivatives possessing bulky substituents at the pyridinium ring (mainly phenyls, *tert*-butyls; *n*-butyl, *n*-propyl or *iso*-propyl), such as **34-37**, **51** and **67**, were very ineffective CA IX inhibitors, showing inhibition constants > 500 nM; (iii) another group of compounds, including **27**, **30-33**, **44**, and **60** showed a moderate inhibitory power towards the tumor-associated isozyme IX, showing K_i values in the range of 160 – 450 nM. Most of these compounds are sulfanilamide derivatives (except **44** and **60**), and the substitution pattern at the pyridinium ring includes (with one exception, **27**) at least one phenyl group in 4, or two phenyls in the 2 and 4 positions. It should be noted that the corresponding homosulfanilamides and 4-aminoethylbenzene-sulfonamides incorporating the same substitution pattern as the compounds mentioned above (sulfanilamides), lead to much better CA IX inhibitors (see later in the text); (iv) a third group of derivatives, including **38**, **45-50**, **52**, **53**, **61**, **63-66**, **68** and **69**, showed good CA IX inhibitory properties, with K_i values in the range of 64 – 135 nM. As mentioned above, except for the tetramethyl-pyridinium-substituted derivative **38**, most of these compounds incorporate 4-phenyl-pyridinium or 2,4-diphenylpyridinium moieties, whereas the group in position 6 is generally quite variable (alkyls or phenyl are tolerated). The most interesting observation regarding this subtype of CA IX inhibitors is constituted by the fact that the 2,4,6-triphenyl-pyridinium- and 2,6-

diphenyl-pyridinium derivatives of homosulfanilamide and 4-aminoethylbenzenesulfonamide (**52-53** and **68-69**) efficiently inhibit isozyme IX, although they act as very weak inhibitors for isozymes I, II and IV (Table 2). As it will be discussed shortly, this may be due to the fact that the hCA IX active site is larger than that of the other investigated isozymes, notably CA II, I and IV; (v) a last group of derivatives (**28-29**; **39-43**; **54**; **55-59**; **62** and **70**) showed very good CA IX inhibitory properties, these compounds possessing K_i values in the range of 6 – 54 nM, similarly to the clinically used inhibitors acetazolamide, methazolamide, dichlorophenamide and indisulam, for which the inhibition data are provided for comparison. It should be noted that three derivatives **58**, **59** and **70** showed inhibition constants < 10 nM, these being the most potent CA IX inhibitors ever reported up to now. Correlated with their membrane-impermeability [96, 85], it may be assumed that *in vivo* such compounds may lead for the first time to a selective CA IX inhibition. Thus, the best substitution pattern at the pyridinium ring includes either only compact alkyls (**39-41**, **54**, **55** and **70**), or 2,6-dialkyl-4-phenyl-pyridinium moieties (all compounds mentioned above except **62**, which incorporates a 2-methyl-4,6-diphenylpyridinium ring); (vi) the number of the substituents at the pyridinium ring seems to be less important for the activity of this series of CAls, since both di-, tri- or tetrasubstituted derivatives showed good inhibitory potency. The nature of these groups on the other hand – as discussed in detail above – is the most important parameter influencing CA inhibitory properties (together with the linker between the benzenesulfonamide moiety and the substituted pyridinium ring); (vii) the isozyme most similar to hCA IX regarding the affinity for these inhibitors was hCA II (which has 33 % homology with hCA IX) [Pastorek et al. (1994), *supra*] whereas the affinities of isozymes I and IV were rather different.

Screening of representative pyridinium derivatives of heterocyclic sulfonamides for inhibition of MN protein, and comparison with inhibition of other CA isozymes: Isozyme I. As seen from data of Table 3, all derivatives **71-91** reported here act as very efficient CAls against this isozyme which is generally the most “resistant” to inhibitors of this type [30, 31, 100, 102]. Indeed, aminobenzolamide is already a highly potent CA I inhibitor (K_i of 6 nM), whereas inhibitors **71-91** show inhibition constants in the range of 3-12 nM, in contrast to the clinically used

sulfonamide CAls which are much less effective inhibitors, with K_i values in the range of 30 - 1200 nM (Table 3). Thus, derivatives possessing several bulky groups (*i*-Pr; *t*-Bu; *n*-Pr; *n*-Bu; Ph, etc) substituting the pyridinium moiety, such as **73, 74, 77, 78, 82, 84, 85** showed a decreased inhibitory activity as compared to

5 aminobenzolamide, with K_i values in the range of 7-12 nM (aminobenzolamide has a K_i of 6 nM against hCA I). The rest of the compounds were more efficient as compared to aminobenzolamide in inhibiting this isozyme, with K_i values in the range of 3-5 nM. Best CA I inhibitors were **75**, and **89-91** (K_i of 3 nM), all of which containing either only alkyl moieties or 4-Ph and other alkyl moieties substituting the

10 pyridinium ring. These are probably the best CA I inhibitors ever reported up to now, since the clinically used CAls show much higher inhibition constants against isozyme I (Table 3).

Isozyme II. Aminobenzolamide is already a very potent CA II inhibitor, with an inhibition constant around 2 nM. Several of the new inhibitors, such as

15 **74,77,78,82-88** act as weaker CA II inhibitors as compared to aminobenzolamide, with K_i values in the range of 3.13-5.96 nM (but all these compounds act as potent inhibitors, being much more effective than the clinically used CAls acetazolamide, methazolamide, dichlorophenamide or indisulam – see Table 3). Again the substitution pattern at the pyridinium ring is the main discriminator of activity for

20 these compounds: all the less active derivatives mentioned above incorporate at least two bulky/long aliphatic groups, mainly in positions 2- and 6- of the pyridinium ring (*n*-Pr; *t*-Bu; *n*-Bu; and Ph). The best CA II inhibitors among derivatives **71-91** were those incorporating more compact 2,6-substituents at the pyridinium ring (such as Me, Et) together with a 4-Me or 4-Phe moiety, or those incorporating only aliphatic

25 such groups, such as **71-73,75,76, 79-81, 89-91**, which showed K_i values in the range of 0.20-1.61 nM (thus, for the best inhibitors a factor of 10 increase in inhibitory power as compared to aminobenzolamide). It should be mentioned that *iso*-propyl-substituted compounds (**73, 79**) are active as CA II inhibitors, although their activity against CA I was not so good.

30 *Isozyme IV.* Most sulfonamides show inhibitory activity against CA IV intermediate between those towards CA I (less susceptible) and CA II (very high affinity for sulfonamides). This is also the trend observed with the sulfonamides

investigated here, derivatives of aminobenzolamide. Thus, the parent sulfonamide (shown in Figure 5) is a potent CA IV inhibitor, with a K_i value around 5 nM. The new derivatives of general formula (B) incorporating bulky pyridinium-ring substituents (such as **74**, **77**, **78**, **82**, **84-88**, **90**) were less effective than aminobenzolamide, showing K_i values in the range of 5.2-10.3 nM, whereas the compounds showing the other substitution pattern mentioned above were better CA IV inhibitors, showing K_i values in the range of 2.0-4.7 nM.

Isozyme IX. Aminobenzolamide is less inhibitory against this isozyme (K_i of 38 nM) as compared to other isozymes discussed above. This behavior is difficult to explain at this point, since no X-ray crystal structure of this isozyme has been reported. A very encouraging result obtained with the new derivatives of general formula (B) reported here, was the observation that several of them show very high affinity for CA IX, with K_i values in the range of 3-9 nM (derivatives **71**, **72**, **75**, **76**, and **89**). It may be seen that all of them incorporate aliphatic moieties (Me, Et and i-Pr) in positions 2- and 6- of the pyridinium ring, and either 4-Me or 4-Ph moieties. Only one compound is tetrasubstituted (**89**), again possessing only methyl moieties. The best CA IX inhibitor (and the best ever reported up to now) was **71**, which is almost 13 times more effective than benzolamide in inhibiting this isozyme. Another group of new derivatives, such as **73**, **74**, **77**, **79**, **80**, **81**, **83**, **86-88**, **90**, **91**, showed effective CA IX inhibition, with K_i values in the range of 12-35 nM, being thus more effective than aminobenzolamide. They incorporate slightly bulkier groups as compared to the previously discussed ones. Again the less effective inhibitors (K_i values in the range of 40-43 nM) were those incorporating several bulky pyridinium substituents, such as **78**, **84**, **85** which contained either two *n*-Bu or one Ph and *n*-Bu/*t*-Bu in positions 2- and 6- of the pyridinium ring. Thus, SAR is now rather clear for this type of CAls: best CA IX inhibitors should contain either only small, compact aliphatic moieties substituting the pyridinium ring, or they tolerate a 4-Ph moiety, but the 2,6-substituents should again be small, compact aliphatic moieties. In this particular case, 2,4,6-trisubstituted-pyridinium derivatives were more effective CA IX inhibitors as compared to the tetrasubstituted derivatives.

Membrane impermeability of Heterocyclic Sulfonamide Inhibitors of CA IX. As seen from data of Table 4 of Example 3, incubation of human red cells (which

contain high concentrations of isozymes I and II, i.e., 150 μ M hCA I and 20 μ M hCA II, but not the membrane-bound CA IV or CA IX) [118] with millimolar concentrations of different sulfonamide inhibitors, such as acetazolamide, or methazolamide, led to saturation of the two isozymes present in erythrocytes with inhibitor, already after short periods of incubation (30 min), whereas for benzolamide or aminobenzolamide, a similar effect is achieved after somehow longer periods (60 min) (Table 4). This is obviously due to the high diffusibility through membranes of the first three inhibitors, whereas benzolamide/aminobenzolamide with a pK_a of 3.2 for the second sulfonamido group [58] 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 **71**, **76**, **89**, **91**, in the same conditions, were detected only in very small amounts within the blood red cells, proving that they were unable to penetrate through the membranes, obviously due to their cationic nature. Even after incubation times as long as one hour (and longer, data not shown), 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 (Table 4). This demonstrates that the proposed approach for achieving membrane impermeability works well for the designed positively-charged sulfonamide CAls of the general formula (**B**) (shown above), since the very small amount of sulfonamide detected may be due to contamination of the lysates with very small amount of membranes.

Design of Membrane-Impermeant Sulfonamide Inhibitors of CA IX

No X-ray crystal structure of isozyme IX is available up to now, in strong contrast with hCA II, for which many X-ray crystal structures are available (alone or in complexes with inhibitors and activators) [1, 2, 14, 15, 19a, 19b, 37, 38]. Examining the active site residues of these two isozymes and the architecture of hCA II, may help explain the above inhibition data and their relevance for CA IX specific inhibitors.

First of all, the zinc ligands and the proton shuttle residue of these two isozymes are identical [33, 43, 72, 100, 101, 102, 114, 115, 117]. An important

difference is constituted by the amino acid in position 131, which is Phe for hCA II and Val for hCA IX. Phe 131 is known to be very important for the binding of sulfonamide inhibitors to hCA II [2, 46, 47]: in many cases this bulky side chain limits the space available for the inhibitor aromatic moieties, or it may participate in stacking interactions with groups present in it (for recent examples see refs. [2, 46, 47]. Thus, the presence of a less bulky such residue in hCA IX (i.e., a valine) which is also unavailable for participation to stacking interactions has as a consequence the fact that the hCA IX active site is larger than that of hCA II. A second potentially important residue is 132, which is Gly in hCA II and Asp in hCA IX. This residue is situated on the rim of the hydrophilic half of the entrance to the active site of hCA II (and presumably also of hCA IX) and it is critical for the interaction with inhibitors possessing elongated molecules, as recently shown by us [19b]. Strong hydrogen bonds involving the CONH moiety of Gly 132 were shown to stabilize the complex of this isozyme with a *p*-aminoethylbenzenesulfonamide derived inhibitor [19b]. In the case of hCA IX, the presence of aspartic acid in this position at the entrance of the active site may signify that: (i) stronger interactions with polar moieties of the inhibitor bound within the active site should be possible, since the COOH moiety possesses more donor atoms; (ii) this residue may have flexible conformations, fine-tuning in this way the interaction with inhibitors. Thus, the stronger hCA IX inhibition with some of these inhibitors (as compared to their affinity for isozyme II), such as for example 46-50, 52, 53, 55, 58, 62 and 68-70, might be explained just by the different interactions with the two active site residues mentioned above.

Therapeutic Use of MN-Specific Inhibitors

The MN-specific inhibitors of this invention, organic and/or inorganic, preferably organic, and as outlined above, may be used therapeutically in the treatment of neoplastic and/or pre-neoplastic disease, either alone or in combination with other chemotherapeutic drugs.

The MN-specific inhibitors can be administered in a therapeutically effective amount, preferably dispersed in a physiologically acceptable, non-toxic liquid vehicle.

Materials and Methods

General. Melting points: heating plate microscope (not corrected); IR spectra: KBr pellets, 400-4000 cm^{-1} Perkin-Elmer*16PC FTIR spectrometer; ^1H -NMR spectra: Varian*300CXP apparatus (chemical shifts are expressed as δ values relative to Me_4Si as standard); Elemental analysis: Carlo Erba*Instrument CHNS Elemental Analyzer, Model 1106. All reactions were monitored by thin-layer chromatography (TLC) using 0.25-mm precoated silica gel plates (E. Merck). Pyrylium salts were prepared by literature procedures, generally by olefin (or their precursors) bisacylation, as described in the literature [6, 26, 108], whereas aminobenzolamide as described earlier [97]. Other sulfonamides used as standards were commercially available.

General procedure for the preparation of compounds 71-91 (Pyridinium derivatives of aminobenzolamide)

An amount of 2.9 mM of aminobenzolamide [97] and 2.9 mM of pyrylium salt II (depicted in Figure 5) 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 hours. 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 the general formula (B) (shown above). In the case of aminobenzolamide, this procedure is the only one which gave acceptable yields in pyridinium salts, probably due to the deactivating effect of the sulfamoylaminothiadiazole 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 .

*trade-marks

Purification of Catalytic Domain of CA IX

The cDNA of the catalytic domain of hCA IX (isolated as described by Pastorek et al. [72]) was amplified by using PCR and specific primers for the vector pCAL-n-FLAG (from Stratagene^{*}). The obtained construct was inserted in the pCAL-n-FLAG vector and then cloned and expressed in *Escherichia coli* strain BL21-GOLD(DE3) (from Stratagene). The bacterial cells were lysed and homogenated in a buffered solution (pH 8) of 4 M urea and 2 % Triton X-100, as described by Wingo et al. [116]. The homogenate thus obtained was extensively centrifuged in order to remove soluble and membrane associated proteins as well as other cellular debris.

The resulting pellet was washed by repeated homogenation and centrifugation in water, in order to remove the remaining urea and Triton X-100. Purified CA IX inclusion bodies were denaturated in 6 M guanidine hydrochloride and refolded into the active form by snap dilution into a solution of 100 mM MES (pH 6), 500 mM L-arginine, 2 mM ZnCl₂, 2 mM EDTA, 2 mM reduced glutathione, 1 mM oxidized glutathione. Active hCA IX was extensively dialysed into a solution of 10mM Hepes (pH 7.5), 10mM Tris HCl, 100mM Na₂SO₄ and 1mM ZnCl₂. The amount of protein was determined by spectrophometric measurements and its activity by stopped-flow measurements, with CO₂ as substrate [44]. Optionally, the protein was further purified by sulfonamide affinity chromatography [44], the amount of enzyme was determined by spectrophometric measurements and its activity by stopped-flow measurements, with CO₂ as substrate [44].

CA I, II and IV purification

Human CA I and CA II cDNAs were expressed in *Escherichia coli* strain BL21 (DE3) from the plasmids pACA/hCA I and pACA/hCA II described by Lindskog's group [54]. Cell growth conditions were those described in ref. [12], and enzymes were purified by affinity chromatography according to the method of Khalifah et al. [45]. Enzyme concentrations were determined spectrophotometrically at 280 nm, utilizing a molar absorptivity of 49 mM⁻¹.cm⁻¹ for CA I and 54 mM⁻¹.cm⁻¹ for CA II, respectively, based on M_r = 28.85 kDa for CA I, and 29.3 kDa for CA II, respectively [53, 84]. CA IV was isolated from bovine lung microsomes as described

* trade-mark

by Maren et al, and its concentration has been determined by titration with ethoxzolamide [59].

Enzyme assays

5 CA CO₂ Hydrase Activity Assay

An SX.18MV-R Applied Photophysics stopped-flow instrument has been used for assaying the CA CO₂ hydration activity assays [44]. A stopped flow variant of the Poker and Stone spectrophotometric method [76] has been employed, using an SX.18MV-R Applied Photophysics stopped flow instrument, as described
10 previously [43]. Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 10 mM Hepes (pH 7.5) as buffer, 0.1 M Na₂SO₄ (for maintaining constant the ionic strength), following the CA-catalyzed CO₂ hydration reaction for a period of 10-100 s. Saturated CO₂ solutions in water at 20 °C were used as substrate [44]. Stock solutions of inhibitor
15 (1 mM) were prepared in distilled-deionized water with 10-20% (v/v) DMSO (which is not inhibitory at these concentrations) 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. Triplicate experiments were done for each
20 inhibitor concentration, and the values reported throughout the paper are the mean of such results.

CA Esterase Activity Assay

Initial rates of 4-nitrophenylacetate hydrolysis catalysed by different CA
25 isozymes were monitored spectrophotometrically, at 400 nm, with a Cary 3 instrument interfaced with an IBM compatible PC [76]. Solutions of substrate were prepared in anhydrous acetonitrile; the substrate concentrations varied between $2 \cdot 10^{-2}$ and $1 \cdot 10^{-6}$ M, working at 25°C. A molar absorption coefficient ϵ of $18,400 \text{ M}^{-1} \cdot \text{cm}^{-1}$ was used for the 4-nitrophenolate formed by hydrolysis, in the conditions of
30 the experiments (pH 7.40), as reported in the literature [76]. Non-enzymatic hydrolysis rates were always subtracted from the observed rates. Triplicate experiments were done for each inhibitor concentration, and the values reported

throughout the paper are the mean of such results. Stock solutions of inhibitor (1-3 mM) were prepared in distilled-deionized water with 10-20% (v/v) DMSO (which is not inhibitory at these concentrations) 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 in references [44, 76].

Membrane Permeance Assay: Ex vivo Penetration through Red Blood Cells

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 - 120 min. After the incubation times of 30, 60 and 120 min., respectively, 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 [81, 96, 98]. 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 (in order to denature CA-s) and sulfonamides possibly present have been assayed in each sample by three methods: a HPLC method [36]; spectrophotometrically [4] and enzymatically [76].

HPLC: A variant of the methods of Gomaa [36] 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; 12.32 min for aminobenzolamide; 3.15 min for 71; 4.41 min for 76; 3.54 min for 89; and 4.24 min for 91. 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).

* trade-marks

Spectrophotometrically: A variant of the pH-induced spectrophotometric assay of Abdine et al. [4] 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 [76]. 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.

Statistical analysis: Values are expressed $\pm$ standard error of measurement. Statistical significance was determined using an unpaired t-test with $p < 0.05$ considered significant.

The following examples are for purposes of illustration only and are not meant to limit the invention in any way.

Example 1

Inhibition of the tumor-associated isozyme IX with aromatic and heterocyclic sulfonamides

The inhibition of the tumor-associated transmembrane carbonic anhydrase IX (CA IX) isozyme has been investigated with a series of aromatic and heterocyclic sulfonamides, including the six clinically used derivatives acetazolamide, methazolamide, ethoxzolamide, dichlorophenamide, dorzolamide and brinzolamide. Inhibition data for the physiologically relevant isozymes I and II (cytosolic forms) and IV (membrane-bound) were also provided for comparison.

Chemistry. Sulfonamides investigated for the inhibition of the tumor-associated isozyme CA IX, of types 1-26 are shown in Figure 4A-B. Compounds 1-6, 11-12, 20 and 26 are commercially available, whereas 7-10 [43], 13-19 [24, 79, 90, 97] and 21-25 [79] were prepared as reported earlier. The six clinically used

compounds were also assayed, since no such data are available in the literature.

CA inhibition data. Inhibition data against four CA isozymes, CA I, II, IV and IX [44, 72, 116], with the above mentioned compounds **1-26** and the six clinically used inhibitors, are shown in Table 1.

5

Table 1: CA I, II, IV and IX inhibition data with sulfonamides **1-26** and clinically used inhibitors.

	Inhibitor	K_i^* (nM)			
		hAC I ^a	hCA II ^a	bCA IV ^b	hCA IX ^c
10	1	45400	295	1310	33
	2	25000	240	2200	238
	3	28000	300	3000	294
	4	78500	320	3215	305
15	5	25000	170	2800	103
	6	21000	160	2450	33
	7	8300	60	180	245
	8	9800	110	320	264
	9	6500	40	66	269
20	10	6000	70	125	285
	11	5800	63	154	24
	12	8400	75	160	39
	13	8600	60	540	41
	14	9300	19	355	30
25	15	6	2	5	38
	16	164	46	129	34
	17	185	50	144	20
	18	109	33	72	31
	19	95	30	72	24
30	20	690	12	154	16
	21	55	8	17	14
	22	21000	125	415	32

(Table 1, continued)

	23	23000	133	438	30
	24	24000	125	560	21
5	25	18000	110	450	22
	26	135	40	86	26
	AAZ	250	12	70	25
	MZA	50	14	36	27
	EZA	25	8	13	34
10	DCP	1200	38	380	50
	DZA	50000	9	43	52
	BRZ	-	3	45	37

15 ^a Human cloned isozymes, esterase assay method [76];

^b Isolated from bovine lung microsomes, esterase assay method [76];

^c Human cloned isozyme, CO₂ hydrase assay method [44, 72, 116].

We report here the first inhibition study of the tumor-associated, transmembrane isozyme CA IX with a series of aromatic and heterocyclic sulfonamides, including also the six clinically used derivatives acetazolamide, methazolamide, ethoxzolamide, dichlorophenamide, dorzolamide and brinzolamide. Inhibition data for the physiologically relevant isozymes I and II (cytosolic forms) and IV (membrane-bound) are also provided for comparison. Very interesting inhibition profile against CA IX with these sulfonamides has been detected, which is a promising discovery for the potential design of CA IX-specific inhibitors, with applications as antitumor agents. Several nanomolar CA IX inhibitors have been detected, both among the aromatic (such as orthanilamide, homosulfanilamide, 4-carboxy-benzenesulfonamide, 1-naphthalene- sulfonamide and 1,3-benzenedisulfonamide derivatives) as well as the heterocyclic (such as 1,3,4-thiadiazole-2-sulfonamide, benzothiazole-2-sulfonamide, etc.) sulfonamides investigated.

Example 2

The first selective, membrane-impermeant inhibitors targeting the tumor-associated isozyme IX

5 Up to now no CA IX inhibition studies with this type of membrane-impermeant CAls have been reported. Thus, we decided to explore some of the pyridinium derivatives of general formula (A) for their interaction with the catalytic domain of tumor-associated isozyme IX, recently cloned and purified by the
10 inventors [33, 43, 114, 115, 117], as well as the cytosolic, physiologically relevant isozymes CA I, II and the membrane-anchored isozyme CA IV [88, 96].

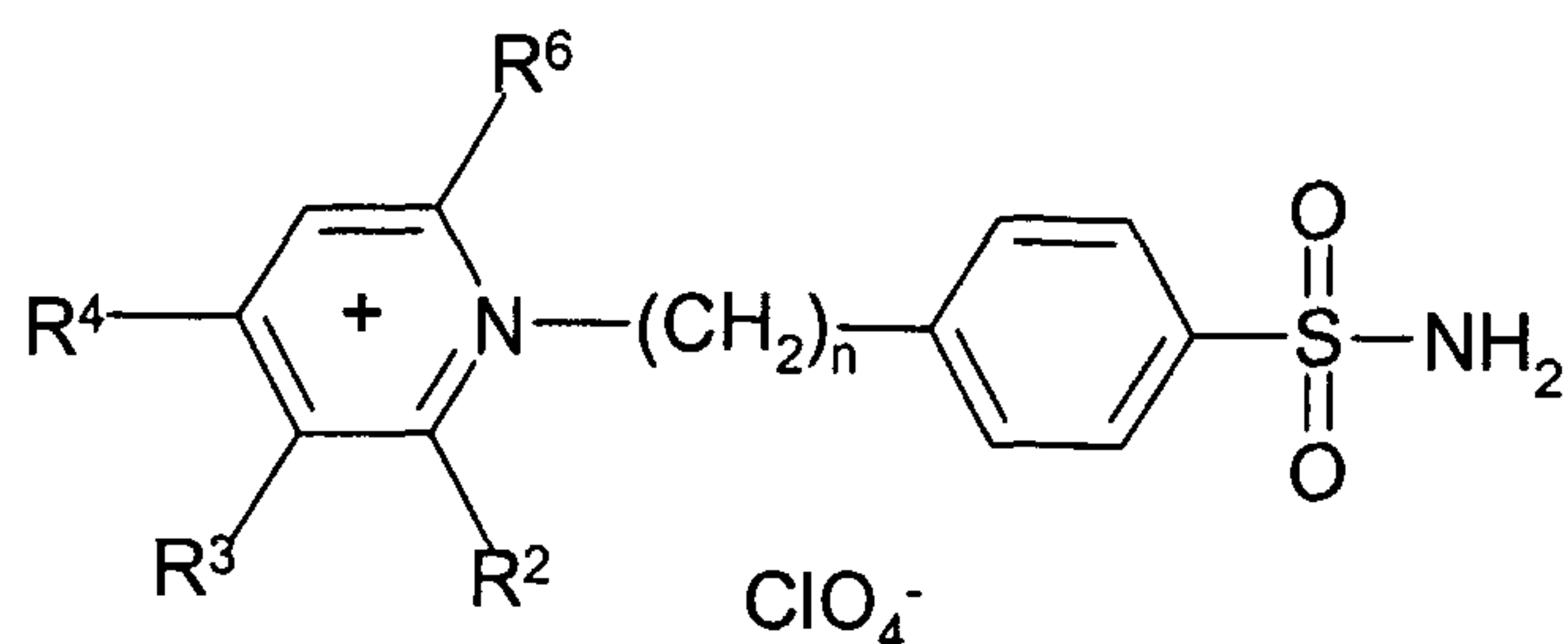
The inhibition of the tumor-associated transmembrane carbonic anhydrase IX (CA IX) isozyme has been investigated with a series of positively-charged, pyridinium derivatives of sulfanilamide, homosulfanilamide and 4-
15 aminoethyl-benzenesulfonamide. Inhibition data for the physiologically relevant isozymes I and II (cytosolic forms) and IV (membrane-bound) were also provided for comparison. This is the first report of inhibitors that may selectively target CA IX, due to their membrane-impermeability and high affinity for this clinically relevant isozyme.

CA inhibition.

20 Data of Table 2 clearly show that most of the compounds **27-70** act as efficient CA IX inhibitors, and that their affinity for this isozyme differs considerably as compared to affinities for the cytosolic isozymes CA I and II, and the other membrane-associated isozyme investigated, CA IV.

25 In a series of substituted-pyridinium derived sulfanilamides, homosulfanilamides and *p*-aminoethylbenzenesulfonamides, a large number of effective hCA IX inhibitors were detected. Some low nanomolar CA IX inhibitors were reported for the first time. Since these compounds are membrane-impermeant due to their salt-like character, and as hCA IX is present on the extracellular side of many
30 tumors with poor clinical prognosis, compounds of this type target specifically this tumor-associated CA isozyme without affecting the cytosolic CAs known to play important physiological functions. Thus, compounds of this type may constitute the basis of new anticancer therapies based on CA inhibitors.

Table 2: Inhibition of isozymes hCA I, hCA II, bCA IV and hCA IX with the pyridinium salts **27-70**.



5

Compound	R ²	R ³	R ⁴	R ⁶	K _i *				
					hCA I ^a (μM)	hCA II ^a (nM)	bCA IV ^b (nM)	hCA IX ^c (nM)	
10	27	Me	H	Me	Me	10	150	290	165
	28	Me	H	Ph	Me	7	60	211	48
	29	Et	H	Ph	Et	6	60	182	43
	30	<i>n</i> -Pr	H	Ph	<i>n</i> -Pr	10	120	194	178
	31	<i>i</i> -Pr	H	Ph	<i>i</i> -Pr	5	50	90	160
15	32	Me	H	Ph	Ph	40	210	852	280
	33	Et	H	Ph	Ph	43	400	1300	450
	34	<i>n</i> -Pr	H	Ph	Ph	140	580	1483	>500
	35	<i>i</i> -Pr	H	Ph	Ph	125	440	2102	>500
	36	<i>n</i> -Bu	H	Ph	Ph	305	620	2155	>500
20	37	Ph	H	Ph	Ph	290	510	2500	>500
	38	Me	Me	Me	Me	5	40	61	72
	39	Me	H	Me	Me	7	50	92	38
	40	<i>i</i> -Pr	H	Me	Me	6	50	80	42
	41	<i>i</i> -Pr	H	Me	<i>i</i> -Pr	11	80	144	54
25	42	Me	H	Ph	Me	4	20	70	26
	43	Et	H	Ph	Et	2	21	52	29
	44	<i>n</i> -Pr	H	Ph	<i>n</i> -Pr	24	90	163	230
	45	<i>i</i> -Pr	H	Ph	<i>i</i> -Pr	12	61	101	100
	46	Me	H	Ph	Ph	32	121	161	64
	47	Et	H	Ph	Ph	42	314	983	79

(Table 2, continued)

	48	<i>n</i> -Pr	H	Ph	Ph	130	390	1260	85
	49	<i>i</i> -Pr	H	Ph	Ph	112	370	1214	80
5	50	<i>n</i> -Bu	H	Ph	Ph	300	595	2104	135
	51	<i>t</i> -Bu	H	Ph	Ph	110	321	1070	>500
	52	Ph	H	Ph	Ph	280	472	1956	120
	53	Ph	H	H	Ph	280	493	1954	106
	54	Me	Me	Me	Me	3	30	51	35
10	55	Me	H	Me	Me	4	21	60	14
	56	<i>i</i> -Pr	H	Me	Me	2	15	32	31
	57	<i>i</i> -Pr	H	Me	<i>i</i> -Pr	3	20	70	49
	58	Me	H	Ph	Me	1	8	20	6
	59	Et	H	Ph	Et	1	9	21	8
15	60	<i>n</i> -Pr	H	Ph	<i>n</i> -Pr	7	42	82	205
	61	<i>i</i> -Pr	H	Ph	<i>i</i> -Pr	6	21	70	89
	62	Me	H	Ph	Ph	18	103	144	37
	63	Et	H	Ph	Ph	40	220	761	70
	64	<i>n</i> -Pr	H	Ph	Ph	112	270	1055	84
20	65	<i>i</i> -Pr	H	Ph	Ph	94	350	864	78
	66	<i>n</i> -Bu	H	Ph	Ph	290	544	2008	120
	67	<i>t</i> -Bu	H	Ph	Ph	92	275	1000	>500
	68	Ph	H	Ph	Ph	270	419	1830	95
	69	Ph	H	H	Ph	265	420	1905	81
25	70	Me	Me	Me	Me	2	10	21	8
	acetazolamide					0.25	12	70	25
	methazolamide					0.05	14	36	27
	dichlorophenamide					1.2	38	380	50
	indisulam					0.03	15	65	24

30

^a Human (cloned) isozymes; ^b From bovine lung microsomes; ^c Catalytic

domain of the human, cloned isozyme.

* errors in the range of $\pm 10\%$ of the reported value, from three different determinations.

For compounds 27-38: n = 0; **39-54:** n = 1; **55-70:** n = 2

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Example 3

Design of selective, membrane-impermeant heterocyclic sulphonamide inhibitors targeting the human tumor-associated isozyme IX

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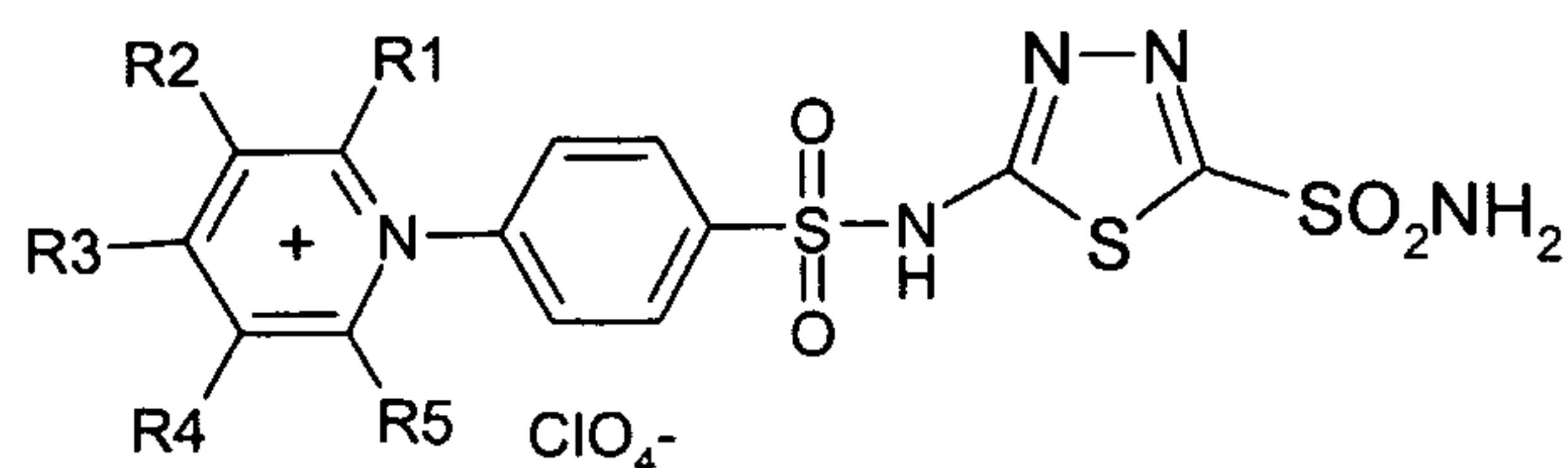
A series of positively-charged sulfonamides were obtained by reaction of aminobenzolamide (5-(4-aminobenzenesulfonylamino)-1,3,4-thiadiazole-2-sulfonamide) with tri-/tetra-substituted pyrilium salts possessing alkyl-, aryl- or combinations of alkyl and aryl groups at the pyridinium ring. These new compounds are membrane-impermeant due to their salt-like character and were assayed for the inhibition of four physiologically relevant carbonic anhydrase (CA, EC 4.2.1.1) isozymes, the cytosolic hCA I and II, the membrane-anchored bCA IV and the membrane-bound, tumor associated isozyme hCA IX. The high affinity of these new derivatives for the tumor-associated isozyme CA IX and their membrane impermeability, make this type of CA inhibitors interesting candidates for the selective inhibition of only the tumor associated isozyme and not the cytosolic ones, for which they also show high potency.

Results

25

CA inhibition. Inhibition data against isozymes I, II, IV and IX with compounds **71-91** reported here are shown in Table 3.

Table 3: Inhibition of isozymes hCA I, hCA II, bCA IV and hCA IX with the pyridinium salts **71-91**.



B

5

		R ¹	R ²	R ³	R ⁴	R ⁵	K _i *(nM)			
		hCA I ^a						hCA II ^a	bCA IV ^b	hCA IX ^c
10	71	Me	H	Me	H	Me	4	0.26	2.1	3
	72	<i>i</i> -Pr	H	Me	H	Me	4	0.39	3.0	5
	73	<i>i</i> -Pr	H	Me	H	<i>i</i> -Pr	7	1.54	4.7	16
	74	<i>t</i> -Bu	H	Me	H	<i>t</i> -Bu	11	3.13	9.4	34
	75	Me	H	Ph	H	Me	3	0.20	2.0	6
15	76	Et	H	Ph	H	Et	4	0.21	2.3	9
	77	<i>n</i> -Pr	H	Ph	H	<i>n</i> -Pr	9	3.45	8.1	35
	78	<i>n</i> -Bu	H	Ph	H	<i>n</i> -Bu	10	4.62	10.3	40
	79	<i>i</i> -Pr	H	Ph	H	<i>i</i> -Pr	5	1.61	4.1	30
	80	Me	H	Ph	H	Ph	4	1.21	3.0	24
20	81	Et	H	Ph	H	Ph	5	1.14	3.8	29
	82	<i>n</i> -Pr	H	Ph	H	Ph	8	3.90	6.0	40
	83	<i>i</i> -Pr	H	Ph	H	Ph	6	3.74	4.5	32
	84	<i>n</i> -Bu	H	Ph	H	Ph	8	4.95	8.4	45
	85	<i>t</i> -Bu	H	Ph	H	Ph	12	4.11	7.0	43
25	86	Ph	H	Me	H	Ph	6	4.78	5.8	12
	87	Ph	H	Ph	H	Ph	5	5.96	5.6	12
	88	Ph	H	H	H	Ph	5	4.93	5.4	16
	89	Me	Me	Me	H	Me	3	0.30	2.4	5
	90	Me	Me	Ph	H	Me	3	1.24	5.2	15
	91	Me	R ³ ,R ⁵ = (CH ₂) ₉ ; R ⁴ =Me			Me	3	1.37	4.6	12

(Table 3, continued)

	aminobenzolamide	6	2.04	5.1	38
	acetazolamide	250	12	70	25
5	methazolamide	50	14	36	27
	dichlorophenamide	1200	38	380	50
	indisulam	30	15	65	24

^a Human (cloned) isozymes, esterase assay method [76].

10 ^b From bovine lung microsomes, esterase assay method [76].

^c Catalytic domain of the human, cloned isozyme, CO₂ hydrase assay method [44].

* Errors in the range of ± 10 % of the reported value, from three different determinations.

15

Ex vivo penetration through red blood cells. Levels of sulfonamides in red blood cells after incubation of human erythrocytes with millimolar solutions of inhibitor for 30-60 min (both classical as well as positively-charged sulfonamides were used in such experiments) are shown in Table 4 [4, 12, 36, 45, 53, 54, 58, 59, 20 84, 116, 118].

Table 4: Levels of sulfonamide CA inhibitors (μM) in red blood cells at 30 and 60 min, after exposure of 10 mL of blood to solutions of sulfonamide (2 mM sulfonamide in 5 mM Tris buffer, pH 7.4). The concentrations of sulfonamide has been determined by three methods: HPLC; electronic spectroscopy (ES) and the enzymatic method (EI) – see Experimental for details.

Inhibitor	[sulfonamide], μM^*					
	HPLC ^a	t = 30 min ES ^b	EI ^c	HPLC ^a	t = 60 min ES ^b	EI ^c
AAZ	136	139	140	160	167	163
MZA	170	169	165	168	168	167
Benzolamide						
	110	108	112	148	146	149
Aminobenzolamide						
	125	127	122	154	156	158
71	0.3	0.5	0.5	0.4	0.5	0.3
76	1.0	1.1	1.0	1.1	1.2	1.1
89	0.3	0.2	0.5	0.3	0.6	0.4
91	0.4	0.3	0.5	0.3	0.6	0.5

* Standard error (from 3 determinations) < 5 % by : ^a the HPLC method [36]; ^b the electronic spectroscopic method [4]; ^c the enzymatic method [76].

The new compounds reported in the present work were characterized by standard chemical and physical methods (elemental analysis, within ± 0.4 % of the theoretical values; IR and NMR spectroscopy) that confirmed their structure (see Materials and Methods and Table 5 below for details) and were assayed for the inhibition of isozymes hCA I, hCA II, bCA IV and hCA IX.

Table 5: Elemental analysis data for the compounds described in Example 3

No	Formula	Elemental analysis data (calc./found)		
		% C	% H	% N
5	71 $C_{16}H_{18}N_5O_4S_3^+ ClO_4^-$	35.59/35.32	3.36/3.62	12.97/12.93
	72 $C_{18}H_{22}N_5O_4S_3^+ ClO_4^-$	38.06/37.95	3.90/4.16	12.33/12.18
	73 $C_{20}H_{26}N_5O_4S_3^+ ClO_4^-$	40.30/39.99	4.40/4.54	11.75/11.63
	74 $C_{22}H_{30}N_5O_4S_3^+ ClO_4^-$	42.34/42.56	4.84/4.76	11.22/11.03
	75 $C_{21}H_{20}N_5O_4S_3^+ ClO_4^-$	41.89/42.02	3.35/3.03	11.63/11.48
10	76 $C_{23}H_{24}N_5O_4S_3^+ ClO_4^-$	43.84/43.88	3.84/3.62	11.11/10.95
	77 $C_{25}H_{28}N_5O_4S_3^+ ClO_4^-$	45.62/45.60	4.29/4.36	10.64/10.50
	78 $C_{27}H_{32}N_5O_4S_3^+ ClO_4^-$	47.26/47.45	4.70/4.89	10.21/10.14
	79 $C_{25}H_{28}N_5O_4S_3^+ ClO_4^-$	45.62/45.49	4.29/4.18	10.64/10.61
	80 $C_{26}H_{22}N_5O_4S_3^+ ClO_4^-$	47.02/46.79	3.34/3.33	10.55/10.23
15	81 $C_{27}H_{24}N_5O_4S_3^+ ClO_4^-$	47.82/47.73	3.57/3.73	10.33/10.40
	82 $C_{28}H_{26}N_5O_4S_3^+ ClO_4^-$	48.59/48.83	3.79/3.91	10.12/10.24
	83 $C_{28}H_{26}N_5O_4S_3^+ ClO_4^-$	48.59/48.27	3.79/3.82	10.12/10.05
	84 $C_{29}H_{28}N_5O_4S_3^+ ClO_4^-$	49.32/49.59	4.00/4.23	9.92/9.67
	85 $C_{29}H_{28}N_5O_4S_3^+ ClO_4^-$	49.32/49.16	4.00/3.94	9.92/9.71
20	86 $C_{26}H_{22}N_5O_4S_3^+ ClO_4^-$	47.02/47.25	3.34/3.18	10.55/10.46
	87 $C_{31}H_{24}N_5O_4S_3^+ ClO_4^-$	51.27/51.50	3.33/3.60	9.64/9.67
	88 $C_{25}H_{20}N_5O_4S_3^+ ClO_4^-$	46.19/46.28	3.10/2.95	10.77/10.67
	89 $C_{17}H_{20}N_5O_4S_3^+ ClO_4^-$	36.86/36.72	3.64/3.53	12.64/12.45
	90 $C_{22}H_{22}N_5O_4S_3^+ ClO_4^-$	42.89/42.70	3.60/3.84	11.37/11.15
25	91 $C_{24}H_{32}N_5O_4S_3^+ ClO_4^-$	44.34/44.57	4.96/4.99	10.77/10.51

Conclusions

We report here a general approach for the preparation of positively-charged, membrane-impermeant sulfonamide CA inhibitors with high affinity for the cytosolic isozymes CA I and CA II, as well as for the membrane-bound ones CA IV and CA IX. They were obtained by attaching substituted-pyridinium moieties to

aminobenzamide, a very potent CA inhibitor itself. Ex vivo studies showed the new class of inhibitors reported here to discriminate for the membrane-bound versus the cytosolic isozymes. Correlated with the low nanomolar affinity of some of these compounds for the tumor-associated isozyme CA IX, this report constitutes the basis of selectively inhibiting only the target, tumor-associated CA IX in vivo, whereas the cytosolic isozymes would remain unaffected.

Characterization of Compounds 71-91 (For preparation, see Materials and Methods Section)

1-N-[5-Sulfamoyl-1,3,4-thiadiazol-2-yl-(aminosulfonyl-4-phenyl)]-2,4,6-trimethyl-pyridinium perchlorate 71: white crystals, mp >300°C; IR (KBr), cm⁻¹ (bands in italics are due to the anion): 595, 625, 664, 787, 803, 884, 915, 1100, 1150, 1190, 1200, 1285, 1360, 1495, 1604, 3065; ¹H-NMR (D₂O), δ, ppm: 3.08 (s, 6H, 2,6-Me₂); 3.11 (s, 3H, 4-Me), 7.30 - 8.06 (m, AA'BB', 4H, ArH from phenylene); 9.05 (s, 2H, ArH, 3,5-H from pyridinium); in this solvent the sulfonamido protons are not seen, being in fast exchange with the solvent. Anal C₁₆H₁₈N₅O₄S₃⁺ ClO₄⁻ (C, H, N).

1-N-[5-Sulfamoyl-1,3,4-thiadiazol-2-yl-(aminosulfonyl-4-phenyl)]-2-iso-propyl-4,6-dimethylpyridinium perchlorate 72, colorless crystals, mp 290-1°C; IR (KBr), cm⁻¹: 625, 680, 720, 1100, 1165, 1330, 1640, 3020, 3235; ¹H-NMR (TFA), δ, ppm: 1.50 (d, 6H, 2Me from *i*-Pr); 2.80 (s, 3H, 6-Me); 2.90 (s, 3H, 4-Me); 3.49 (heptet, 1H, CH from *i*-Pr); 7.25 - 8.43 (m, AA'BB', 4H, ArH from 1,4-phenylene); 7.98 (s, 2H, ArH, 3,5-H from pyridinium). Anal C₁₈H₂₂N₅O₄S₃⁺ ClO₄⁻ (C, H, N).

1-N-[5-Sulfamoyl-1,3,4-thiadiazol-2-yl-(aminosulfonyl-4-phenyl)]-2,6-di-iso-propyl-4-methylpyridinium perchlorate 73, tan crystals, mp 278-9°C; IR (KBr), cm⁻¹: 625, 685, 820, 1100, 1165, 1340, 1635, 3030, 3250; ¹H-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); 7.31 - 8.51 (m, AA'BB', 4H, ArH from 1,4-phenylene); 8.05 (s, 2H, ArH, 3,5-H from pyridinium). Anal C₂₀H₂₆N₅O₄S₃⁺ ClO₄⁻ (C, H, N).

1-N-[5-Sulfamoyl-1,3,4-thiadiazol-2-yl-(aminosulfonyl-4-phenyl)]-2,6-dimethyl-4-phenylpyridinium perchlorate 75, white crystals, mp > 300 °C; IR

(KBr), cm^{-1} : 625, 690, 770, 1100, 1170, 1330, 1635, 3030, 3260, 3330; $^1\text{H-NMR}$ (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 $\text{C}_{21}\text{H}_{20}\text{N}_5\text{O}_4\text{S}_3^+ \text{ClO}_4^-$ (C, H, N).

1-N-[5-Sulfamoyl-1,3,4-thiadiazol-2-yl-(aminosulfonyl-4-phenyl)]-

5 *2,6-diethyl-4-phenylpyridinium perchlorate 76*, tan crystals, mp 267-8 °C; IR (KBr), cm^{-1} : 625, 695, 765, 1100, 1180, 1340, 1630, 3040, 3270, 3360; $^1\text{H-NMR}$ (TFA), δ , ppm: 1.43 (t, 6H, 2 Me from ethyl); 2.82 (q, 4H, 2 CH₂ from Et); 7.68 - 8.87 (m, 11H, ArH from 1,4-phenylene, pyridinium and 4-Ph). Anal $\text{C}_{23}\text{H}_{24}\text{N}_5\text{O}_4\text{S}_3^+ \text{ClO}_4^-$ (C, H, N).

1-N-[5-Sulfamoyl-1,3,4-thiadiazol-2-yl-(aminosulfonyl-4-phenyl)]-

10 *2,6-di-n-propyl-4-phenylpyridinium perchlorate 77*, colorless crystals, mp 235-7 °C; IR (KBr), cm^{-1} : 625, 695, 770, 1100, 1180, 1340, 1630, 3050, 3220, 3315; $^1\text{H-NMR}$ (TFA), δ , ppm: 1.06 (t, 6H, 2 Me from propyl); 1.73 (sextet, 4H, 2CH₂ (β) from *n*-Pr); 2.84 (t, 4H, 2 CH₂ (α) from *n*-Pr); 7.55 - 8.71 (m, 11H, ArH from 1,4-phenylene, pyridinium and 4-Ph). Anal $\text{C}_{25}\text{H}_{28}\text{N}_5\text{O}_4\text{S}_3^+ \text{ClO}_4^-$ (C, H, N).

1-N-[5-Sulfamoyl-1,3,4-thiadiazol-2-yl-(aminosulfonyl-4-phenyl)]-

15 *2,6-di-isopropyl-4-phenylpyridinium perchlorate 79*, white crystals, mp 278-9 °C; IR (KBr), cm^{-1} : 625, 690, 765, 1100, 1180, 1340, 1625, 3040, 3270, 3315; $^1\text{H-NMR}$ (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 $\text{C}_{25}\text{H}_{28}\text{N}_5\text{O}_4\text{S}_3^+ \text{ClO}_4^-$ (C, H, N).

1-N-[5-Sulfamoyl-1,3,4-thiadiazol-2-yl-(aminosulfonyl-4-phenyl)]-2-

25 *methyl-4,6-diphenylpyridinium perchlorate 80*, white crystals, mp 298-99 °C; IR (KBr), cm^{-1} : 625, 710, 770, 1100, 1170, 1345, 1625, 3040, 3245, 3350; $^1\text{H-NMR}$ (TFA), δ , ppm: 2.75 (s, 3H, 2-Me); 7.53 - 8.70 (m, 16H, ArH from 1,4-phenylene, pyridinium and 4,6-Ph₂). Anal $\text{C}_{26}\text{H}_{22}\text{N}_5\text{O}_4\text{S}_3^+ \text{ClO}_4^-$ (C, H, N).

1-N-[5-Sulfamoyl-1,3,4-thiadiazol-2-yl-(aminosulfonyl-4-phenyl)]-2-

30 *ethyl-4,6-diphenylpyridinium perchlorate 81*, white crystals, mp 254-5 °C; IR (KBr), cm^{-1} : 625, 700, 770, 1100, 1180, 1340, 1620, 3040, 3250, 3350; $^1\text{H-NMR}$ (TFA), δ , ppm: 1.52 (t, 3H, Me from ethyl); 2.97 (q, 2H, CH₂); 7.40 - 8.57 (m, 16H, ArH from 1,4-phenylene, pyridinium and 4,6-Ph₂). Anal $\text{C}_{27}\text{H}_{24}\text{N}_5\text{O}_4\text{S}_3^+ \text{ClO}_4^-$ (C, H, N).

1-N-[5-Sulfamoyl-1,3,4-thiadiazol-2-yl-(aminosulfonyl-4-phenyl)]-2-n-propyl-4,6-diphenylpyridinium perchlorate 82, white crystals, mp 214-5 °C; IR (KBr), cm^{-1} : 625, 700, 770, 1100, 1180, 1340, 1620, 3030, 3270, 3350; $^1\text{H-NMR}$ (TFA), δ , ppm: 1.03 (t, 3H, Me from propyl); 1.95 (sextet, 2H, β -CH₂ from *n*-Pr);
 5 2.88 (t, 2H, α -CH₂ from *n*-Pr); 7.39 - 8.55 (m, 16H, ArH from 1,4-phenylene, pyridinium and 4,6-Ph₂). Anal C₂₈H₂₆N₅O₄S₃⁺ ClO₄⁻ (C, H, N).

1-N-[5-Sulfamoyl-1,3,4-thiadiazol-2-yl-(aminosulfonyl-4-phenyl)]-2-iso-propyl-4,6-diphenylpyridinium perchlorate 83, white crystals, mp 186-8 °C; IR (KBr), cm^{-1} : 625, 700, 770, 1100, 1170, 1340, 1620, 3040, 3250, 3360; $^1\text{H-NMR}$
 10 (TFA), δ , ppm: 1.51 (d, 6H, 2 Me from *i*-propyl); 2.50 - 3.27 (m, 1H, CH from *i*-Pr); 7.32 - 8.54 (m, 16H, ArH from 1,4-phenylene, pyridinium and 4,6-Ph₂). Anal C₂₈H₂₆N₅O₄S₃⁺ ClO₄⁻ (C, H, N).

1-N-[5-Sulfamoyl-1,3,4-thiadiazol-2-yl-(aminosulfonyl-4-phenyl)]-2-n-butyl-4,6-diphenylpyridinium perchlorate 84, white crystals, mp 241-3 °C; IR
 15 (KBr), cm^{-1} : 625, 710, 770, 1100, 1180, 1335, 1625, 3040, 3260, 3345; $^1\text{H-NMR}$ (TFA), δ , ppm: 0.93 (t, 3H, Me from butyl); 1.12 - 2.14 (m, 4H, CH₃-CH₂-CH₂-CH₂ from *n*-Bu); 2.96 (t, 2H, α -CH₂ from *n*-Bu); 7.21 - 8.50 (m, 16H, ArH from 1,4-phenylene, pyridinium and 4,6-Ph₂). Anal C₂₉H₂₈N₅O₄S₃⁺ ClO₄⁻ (C, H, N).

1-N-[5-Sulfamoyl-1,3,4-thiadiazol-2-yl-(aminosulfonyl-4-phenyl)]-2-tert-butyl-4,6-diphenylpyridinium perchlorate 85, white crystals, mp 203-5°C; IR
 20 (KBr), cm^{-1} : 625, 705, 765, 1100, 1160, 1310, 1620, 3060, 3270; $^1\text{H-NMR}$ (TFA), δ , ppm: 1.91 (s, 9H, *t*-Bu); 6.80 - 8.74 (m, 16H, ArH from 1,4-phenylene, 4,6-Ph₂ and 3,5-H from pyridinium). Anal C₂₉H₂₈N₅O₄S₃⁺ ClO₄⁻ (C, H, N).

1-N-[5-Sulfamoyl-1,3,4-thiadiazol-2-yl-(aminosulfonyl-4-phenyl)]-2,4,6-triphenyl-pyridinium perchlorate 87: pale yellow crystals, mp>300°C; IR (KBr), cm^{-1}
 25 (bands in italics are due to the anion): 625, 635, 703, 785, 896, *1100*, 1150, 1204, 1355, 1410, 1520, 1600, 3065; $^1\text{H-NMR}$ (D₂O), δ , ppm: 7.50-8.60 (m, 19H, ArH, 3Ph + C₆H₄); 9.27 (s, 2H, ArH, 3,5-H from pyridinium); in this solvent the sulfonamido protons are not seen, being in fast exchange with the solvent. Anal C₃₁H₂₄N₅O₄S₃⁺
 30 ClO₄⁻ (C, H, N).

1-N-[5-Sulfamoyl-1,3,4-thiadiazol-2-yl-(aminosulfonyl-4-phenyl)]-2,6-diphenylpyridinium perchlorate 88, yellow crystals, mp 218-20°C; IR (KBr),

cm⁻¹: 625, 705, 765, 1100, 1160, 1335, 1615, 3050, 3260; ¹H-NMR (TFA), δ, ppm: 6.75 - 8.43 (m, 17H, ArH from 1,4-phenylene, 2,6-Ph₂ and 3,4,5-H from pyridinium). Anal C₂₅H₂₀N₅O₄S₃⁺ ClO₄⁻ (C, H, N).

1-N-[5-Sulfamoyl-1,3,4-thiadiazol-2-yl-(aminosulfonyl-4-phenyl)]-

5 *2,3,4,6-tetramethylpyridinium perchlorate 89*, tan crystals, mp > 300°C; IR (KBr), cm⁻¹: 625, 800, 1100, 1165, 1330, 1630, 3030, 3305; ¹H-NMR (TFA), δ, ppm: 2.62 (s, 3H, 4-Me); 2.74 (s, 3H, 3-Me); 2.88 (s, 6H, 2,6-(Me)₂); 7.21 - 8.50 (m, AA'BB', 4H, ArH from 1,4-phenylene); 7.93 (s, 1H, ArH, 5-H from pyridinium). Anal C₁₇H₂₀N₅O₄S₃⁺ ClO₄⁻ (C, H, N).

10 The description of the foregoing embodiments of the invention have been presented for purposes of illustration and description. They are not intended to be exhaustive or to limit the invention to the precise form disclosed, and obviously many modifications and variations are possible in light of the above teachings. The embodiments were chosen and described in order to explain the principles of the
15 invention and its practical application to enable thereby others skilled in the art to utilize the invention in various embodiments and with various modifications as are suited to the particular use contemplated.

SEQUENCE LISTING

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<120> CA IX-SPECIFIC INHIBITORS

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65	70	75
Ala Pro Gly Asp Pro Gln Glu Pro Gln Asn Asn Ala His Arg Asp Lys		
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Glu Gly Asp Asp Gln Ser His Trp Arg Tyr Gly Gly Asp Pro Pro Trp		
100	105	110
Pro Arg Val Ser Pro Ala Cys Ala Gly Arg Phe Gln Ser Pro Val Asp		
115	120	125
Ile Arg Pro Gln Leu Ala Ala Phe Cys Pro Ala Leu Arg Pro Leu Glu		
130	135	140
Leu Leu Gly Phe Gln Leu Pro Pro Leu Pro Glu Leu Arg Leu Arg Asn		
145	150	155
Asn Gly His Ser Val Gln Leu Thr Leu Pro Pro Gly Leu Glu Met Ala		
165	170	175
Leu Gly Pro Gly Arg Glu Tyr Arg Ala Leu Gln Leu His Leu His Trp		
180	185	190
Gly Ala Ala Gly Arg Pro Gly Ser Glu His Thr Val Glu Gly His Arg		
195	200	205
Phe Pro Ala Glu Ile His Val Val His Leu Ser Thr Ala Phe Ala Arg		
210	215	220

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Ser Arg Leu Glu Glu Ile Ala Glu Glu Gly Ser Glu Thr Gln Val Pro
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Gln Tyr Glu Gly Ser Leu Thr Thr Pro Pro Cys Ala Gln Gly Val Ile
 290 295 300

Trp Thr Val Phe Asn Gln Thr Val Met Leu Ser Ala Lys Gln Leu His
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Thr Leu Ser Asp Thr Leu Trp Gly Pro Gly Asp Ser Arg Leu Gln Leu
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Asn Phe Arg Ala Thr Gln Pro Leu Asn Gly Arg Val Ile Glu Ala Ser
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Phe Leu Val Gln
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<400> 9

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1 5 10 15

Pro Ala Glu Val Ala Glu Thr Gly Ala
20 25

CLAIMS

1. A method that is diagnostic or diagnostic and prognostic for precancer or cancer characterized by overexpression of MN/CA IX protein, comprising contacting a mammalian sample with a potent MN/CA IX-specific inhibitor conjugated to a label or a visualizing means, and detecting or detecting and quantifying binding of said potent MN/CA IX-specific inhibitor to MN/CA IX activated by hypoxic conditions in said sample by detecting or detecting and quantifying said label or said visualizing means on cells in said sample, wherein said detection or said detection and quantitation at a level above that for a control sample is indicative of hypoxic precancerous or cancerous cells that overexpress activated MN/CA IX in said sample;

wherein said inhibitor is selected from the group consisting of organic heterocyclic and aromatic compounds, and wherein said inhibitor is considered to be a potent inhibitor of MN/CA IX enzymatic activity if it is tested in a screening assay that comprises determining the inhibition constant K_i of said compound;

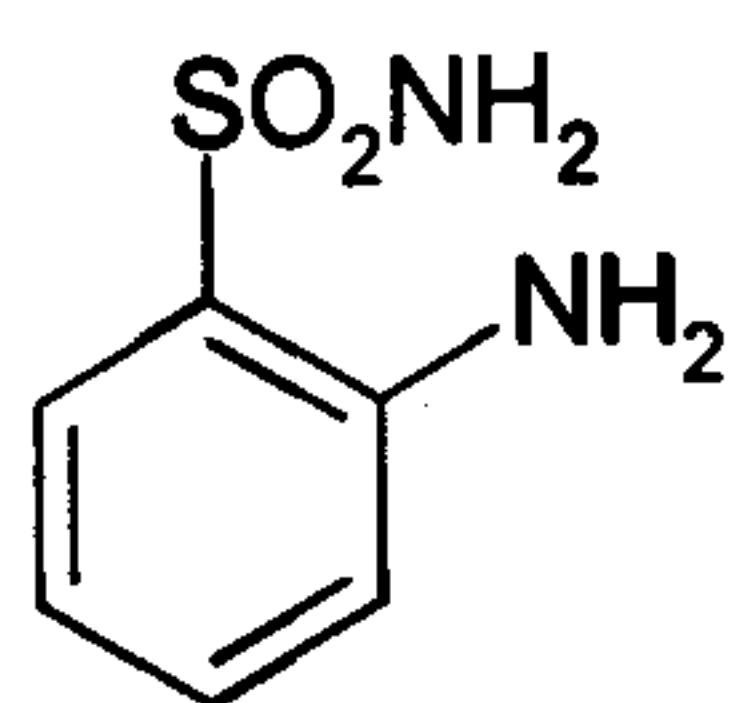
wherein if said inhibition constant K_i is less than 50 nanomolar, said inhibitor is considered to be a potent inhibitor of MN/CA IX enzymatic activity; and

wherein said potent inhibitor is considered to be an MN/CA IX-specific inhibitor if it is a more potent inhibitor of MN/CA IX enzymatic activity than of the enzymatic activity of each of the carbonic anhydrases in a group consisting of CA I, CA II and CA IV.

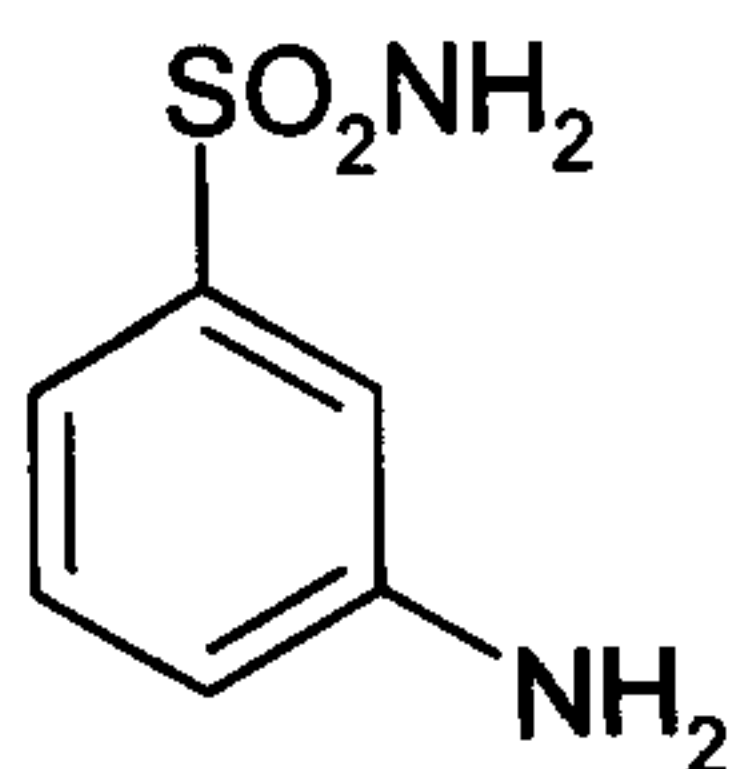
2. The method of claim 1 wherein MN/CA IX activated by hypoxic conditions is detected or detected and quantitated, and the mammal from whom the sample was taken is considered to have a poorer prognosis than if said label or said visualizing means is not detected, and decisions on treatment for said mammal are made in view of the presence of said hypoxic conditions.

3. The method of claim 1 wherein said MN/CA IX-specific inhibitor is an aromatic sulfonamide or a heterocyclic sulfonamide.

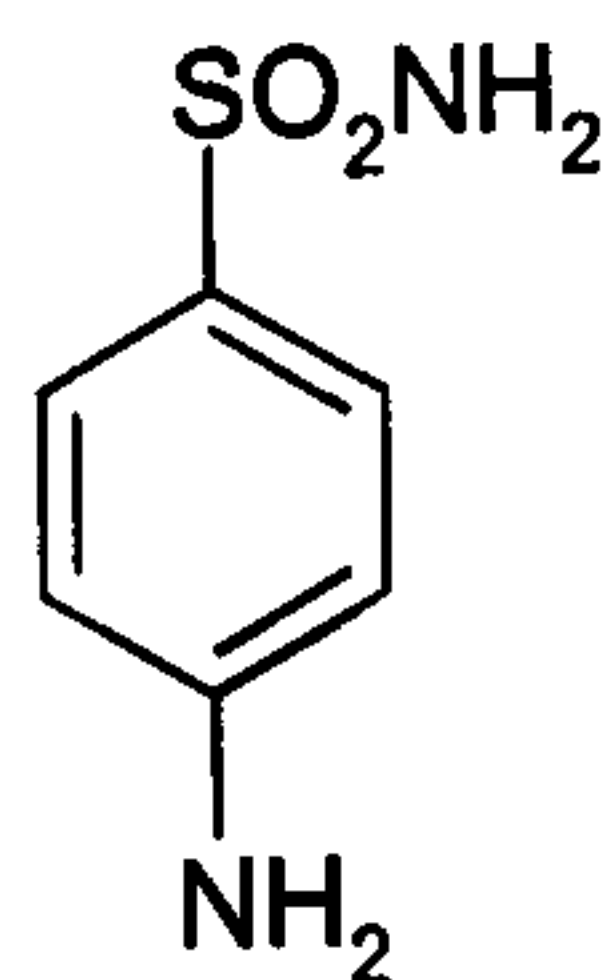
4. The method of claim 1 wherein said MN/CA IX-specific inhibitor is selected from the group consisting of:



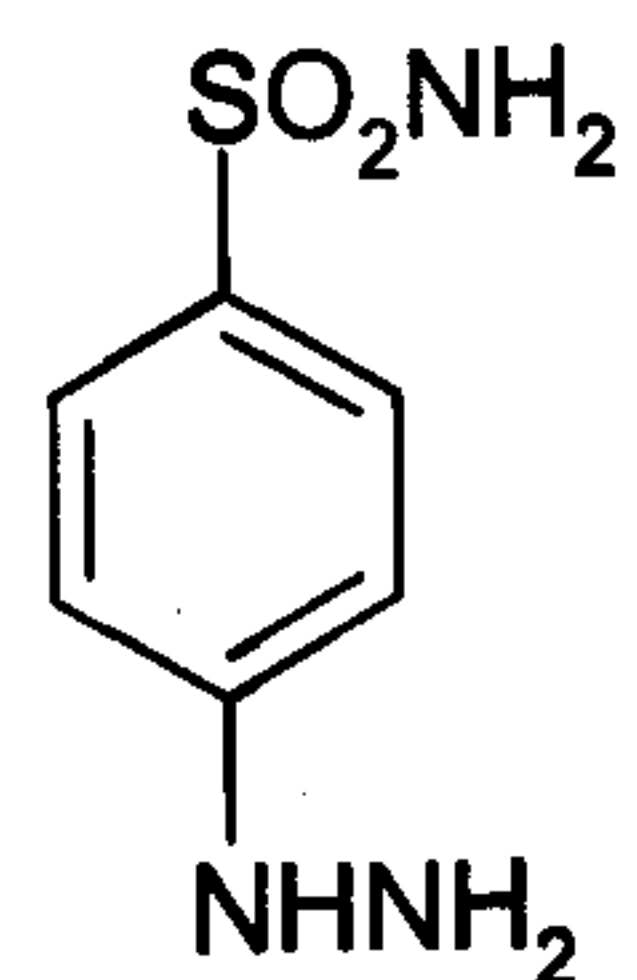
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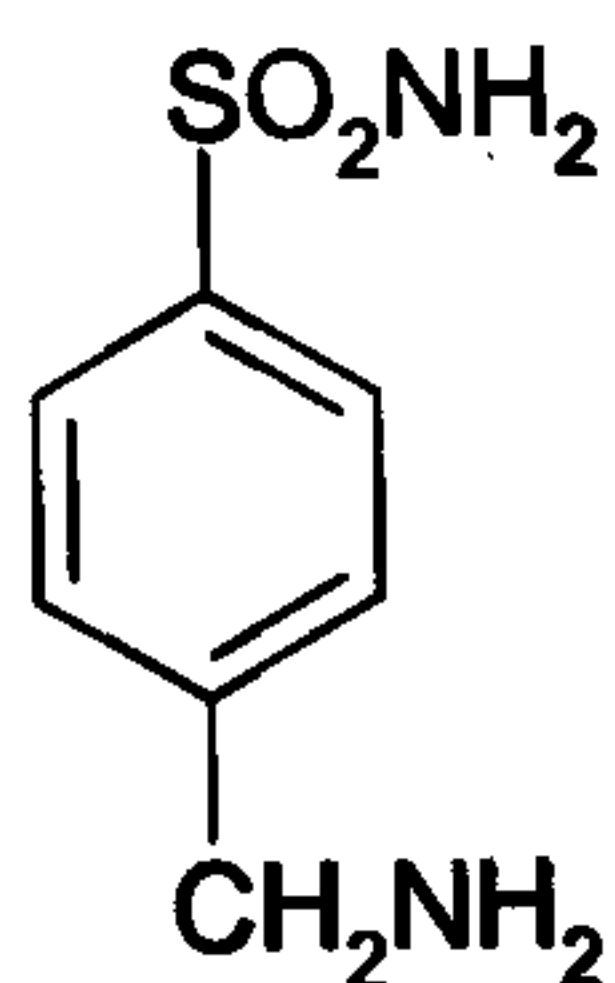
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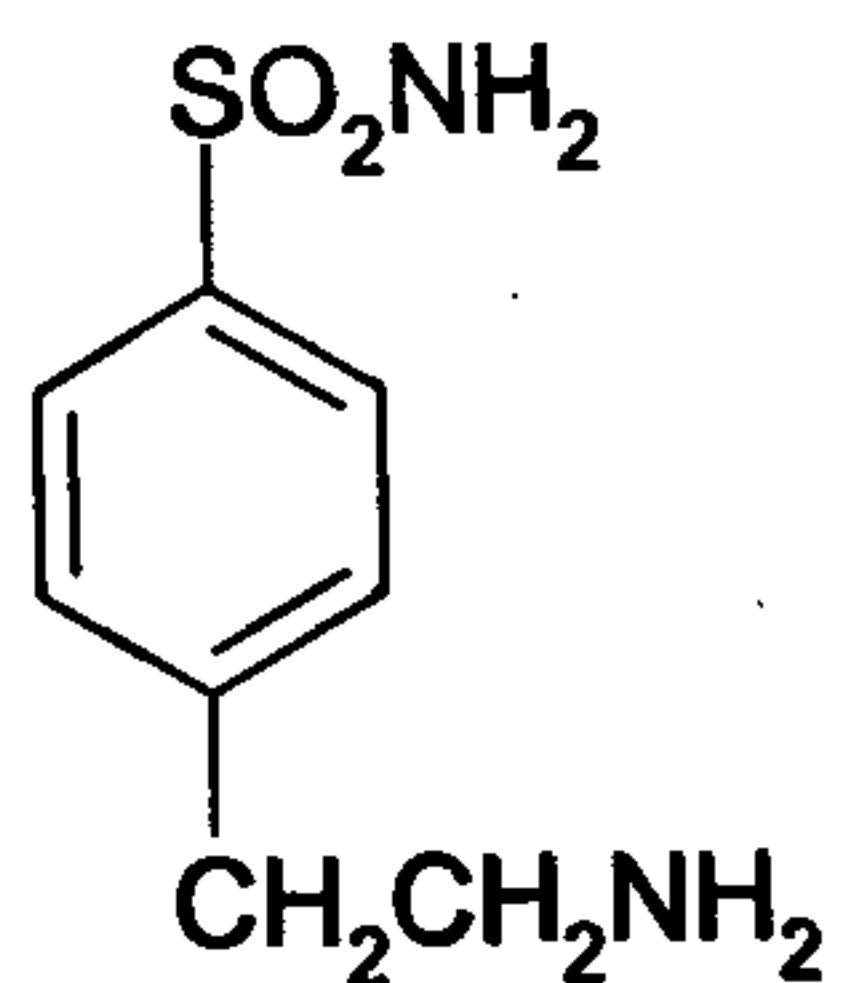
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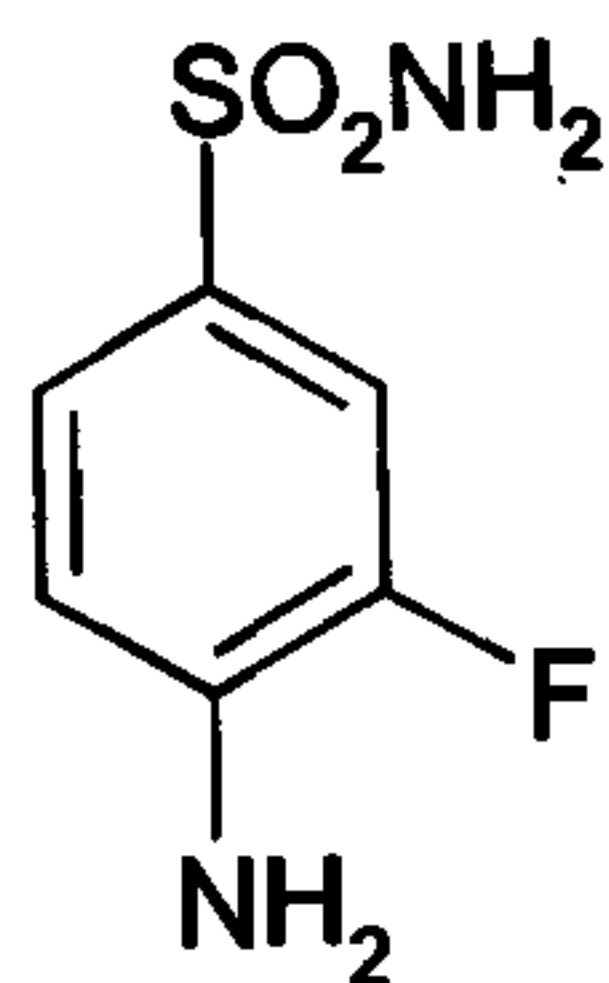
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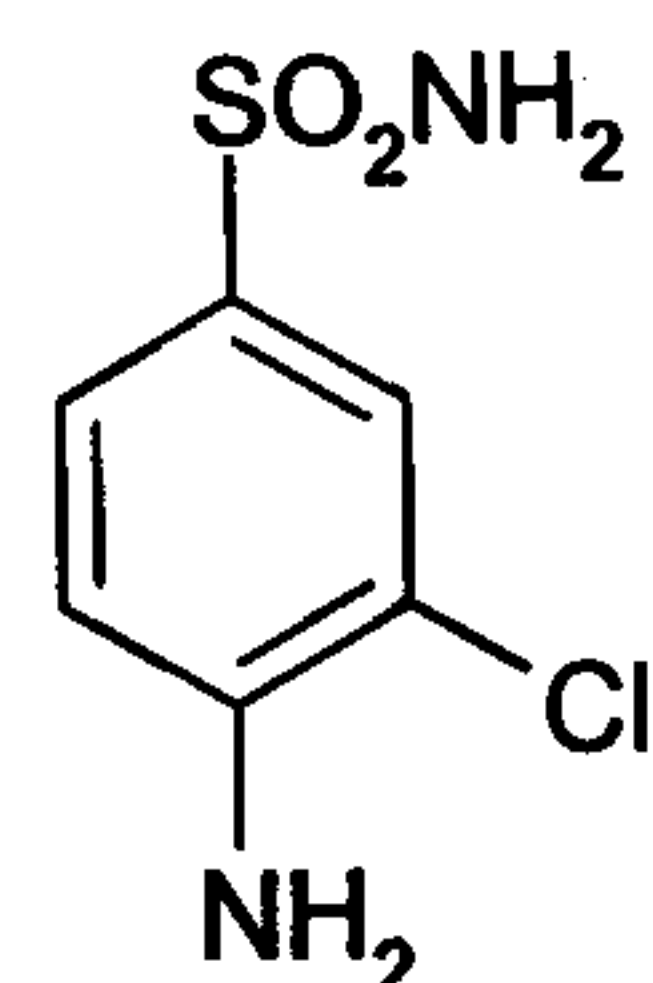
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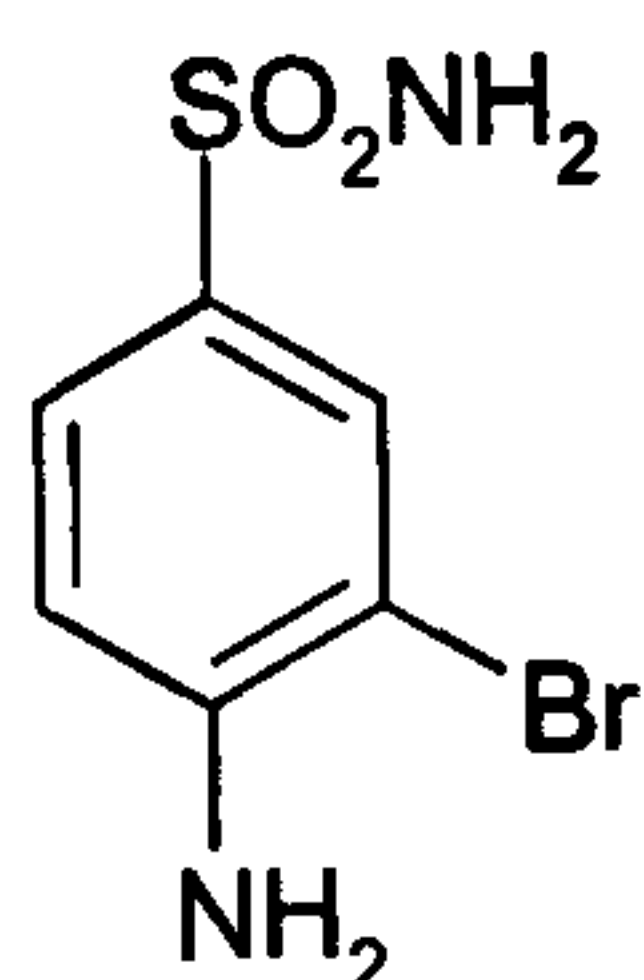
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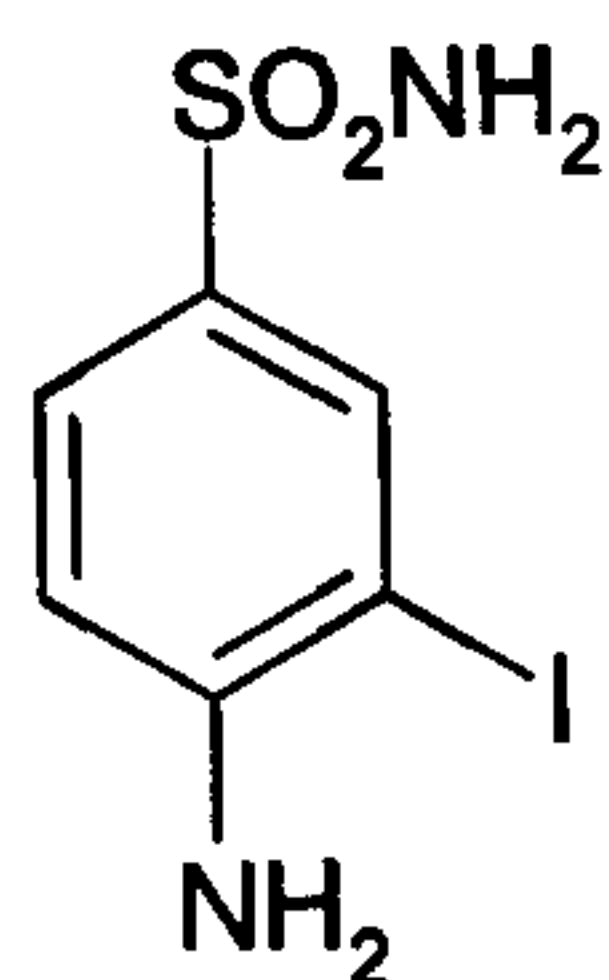
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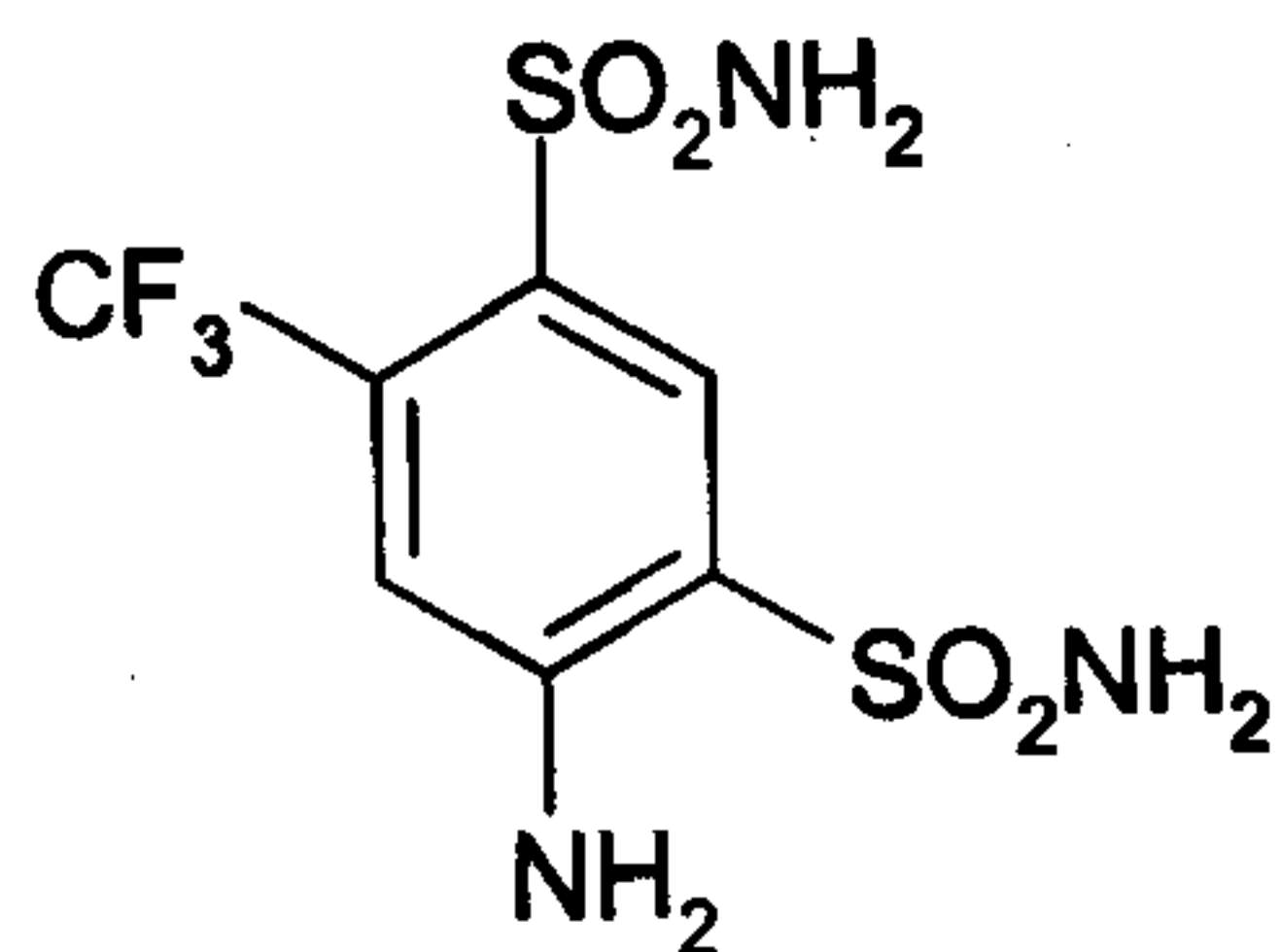
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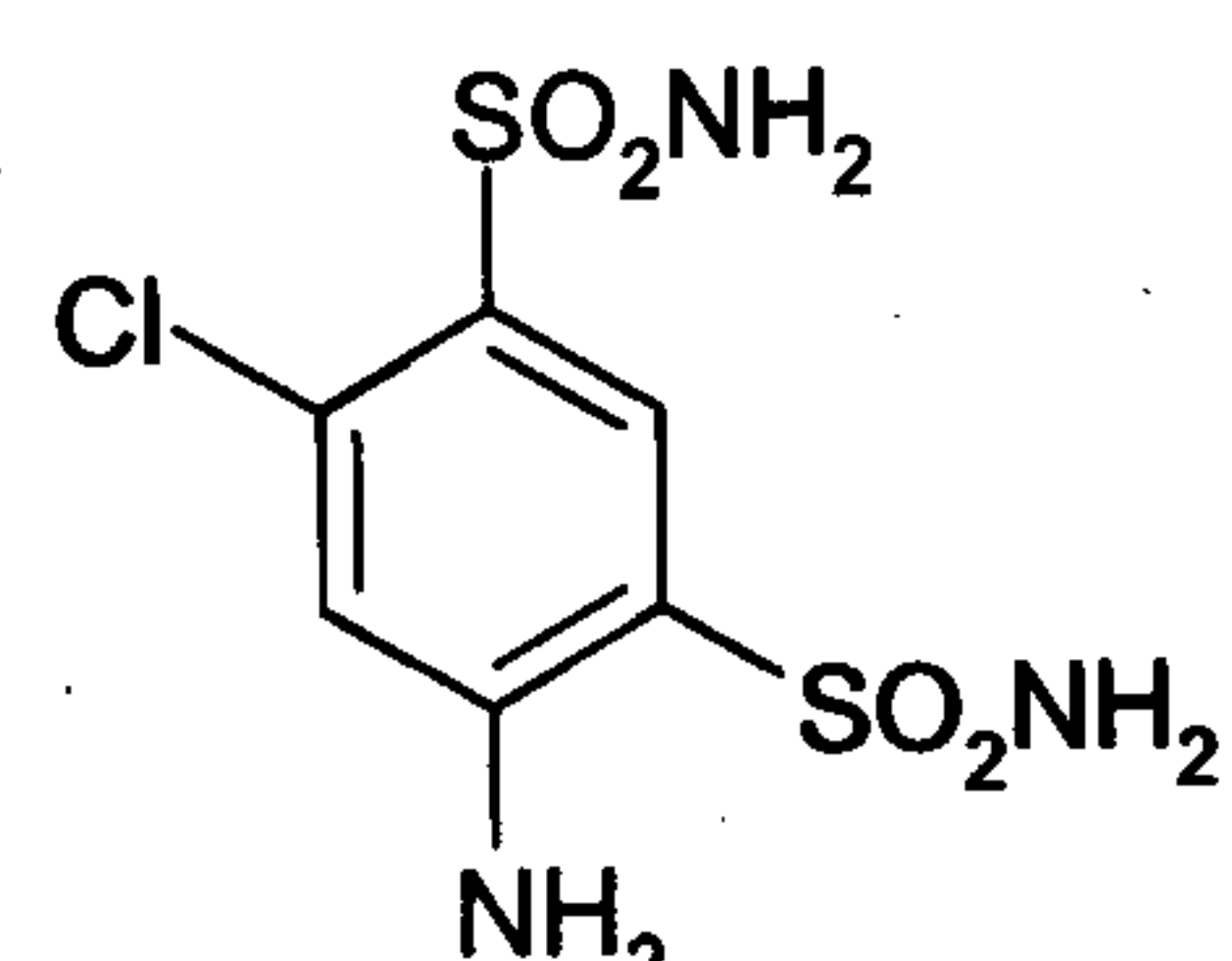
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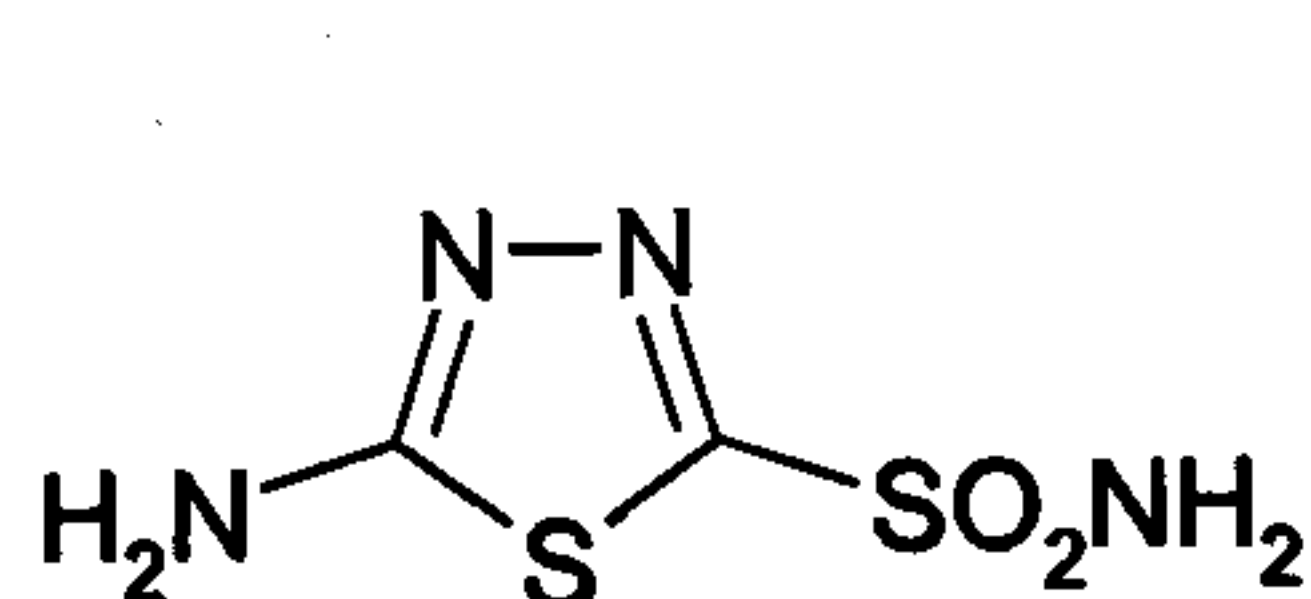
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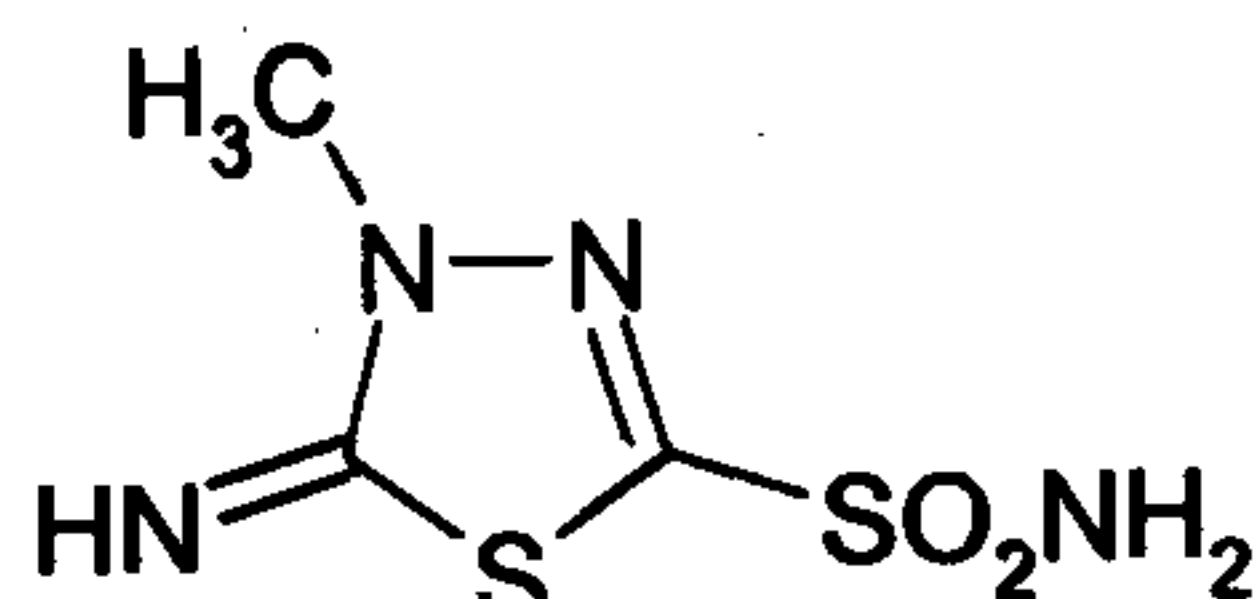
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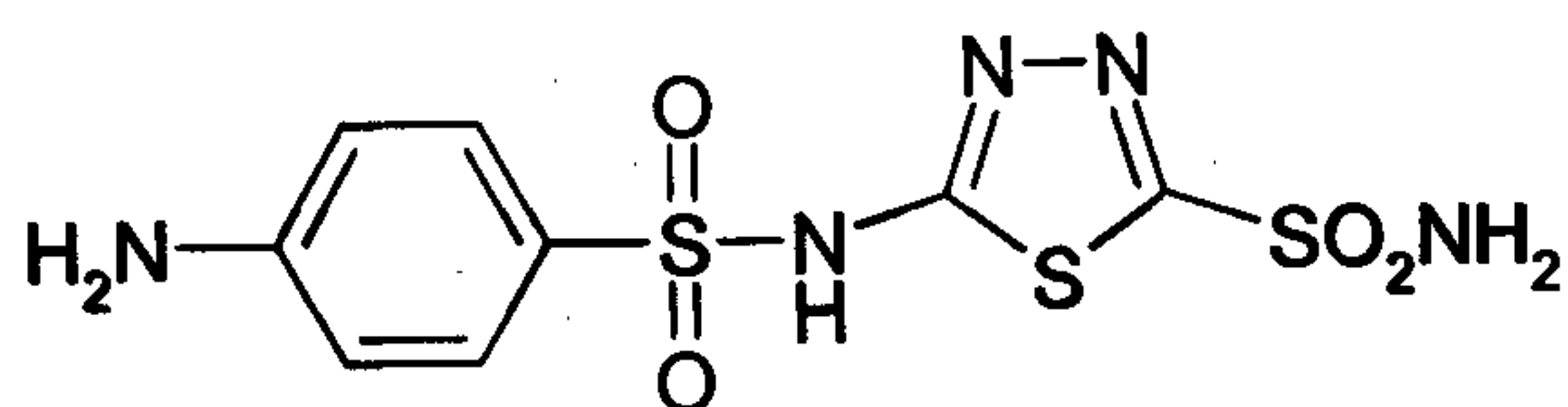
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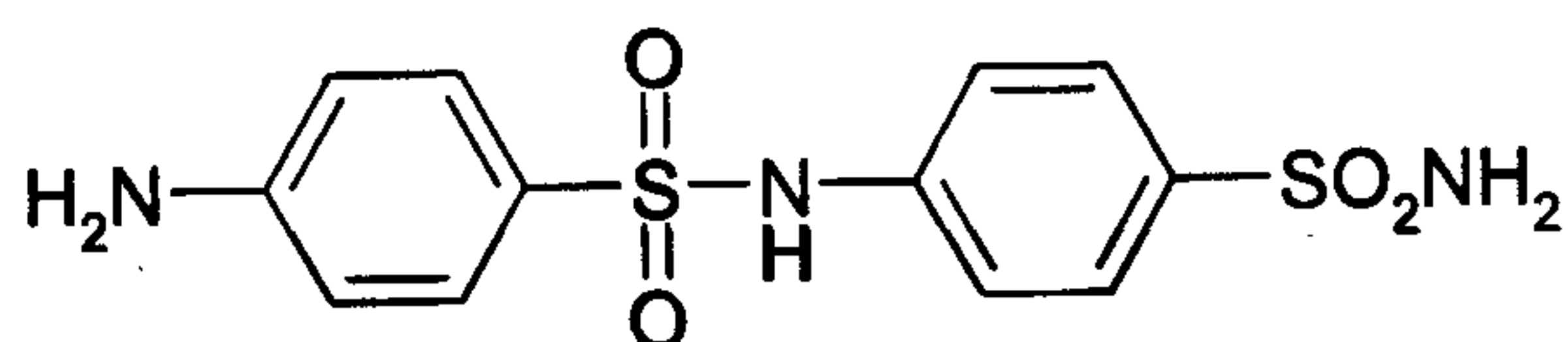
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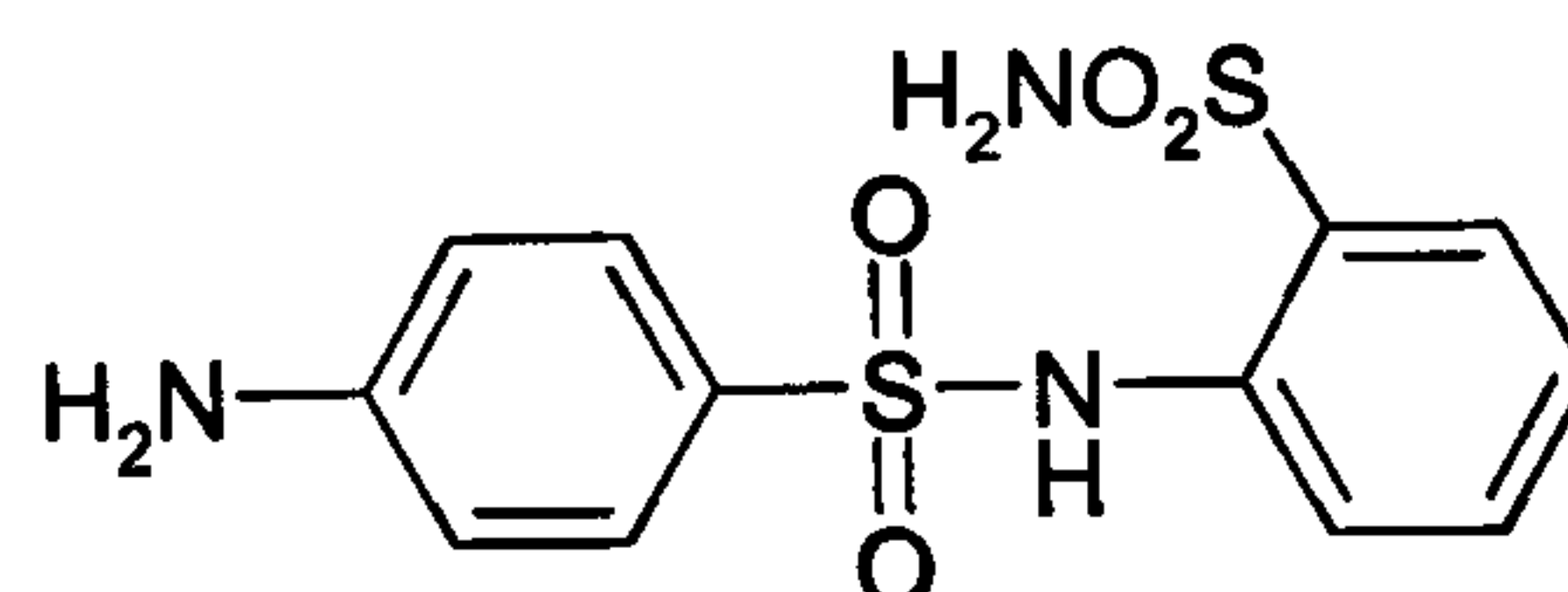
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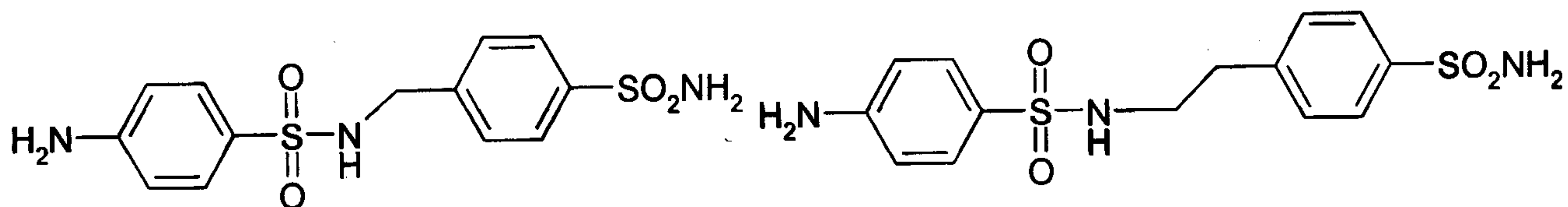
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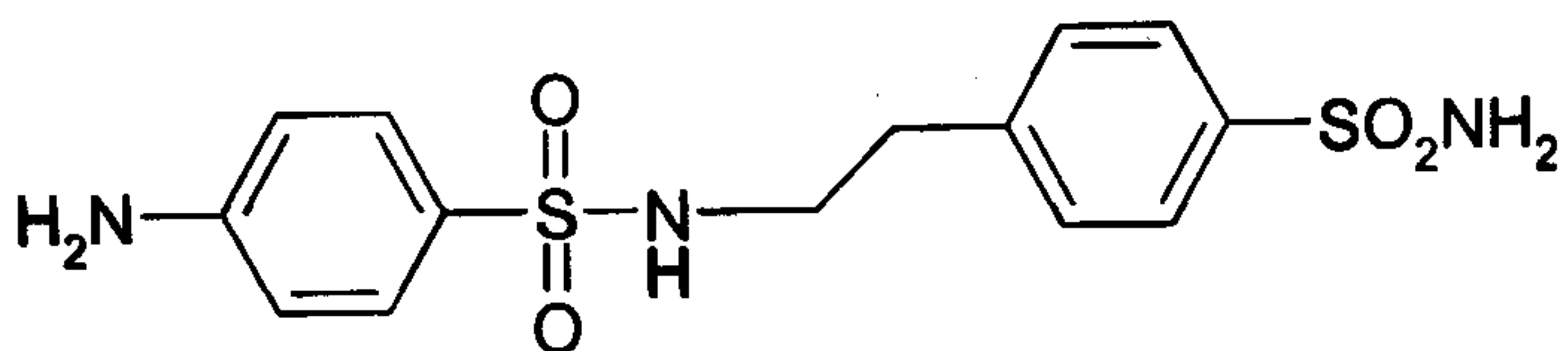
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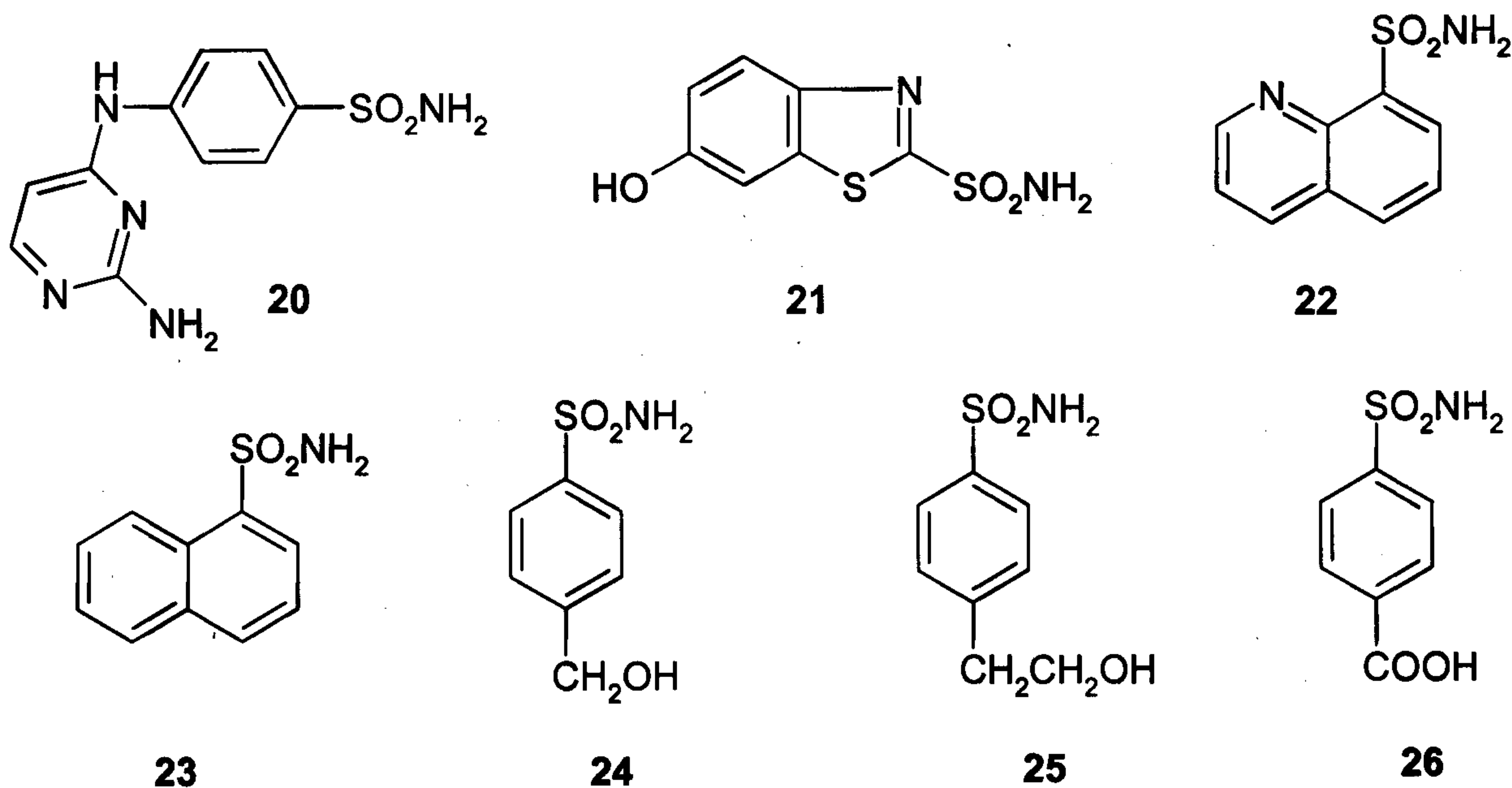
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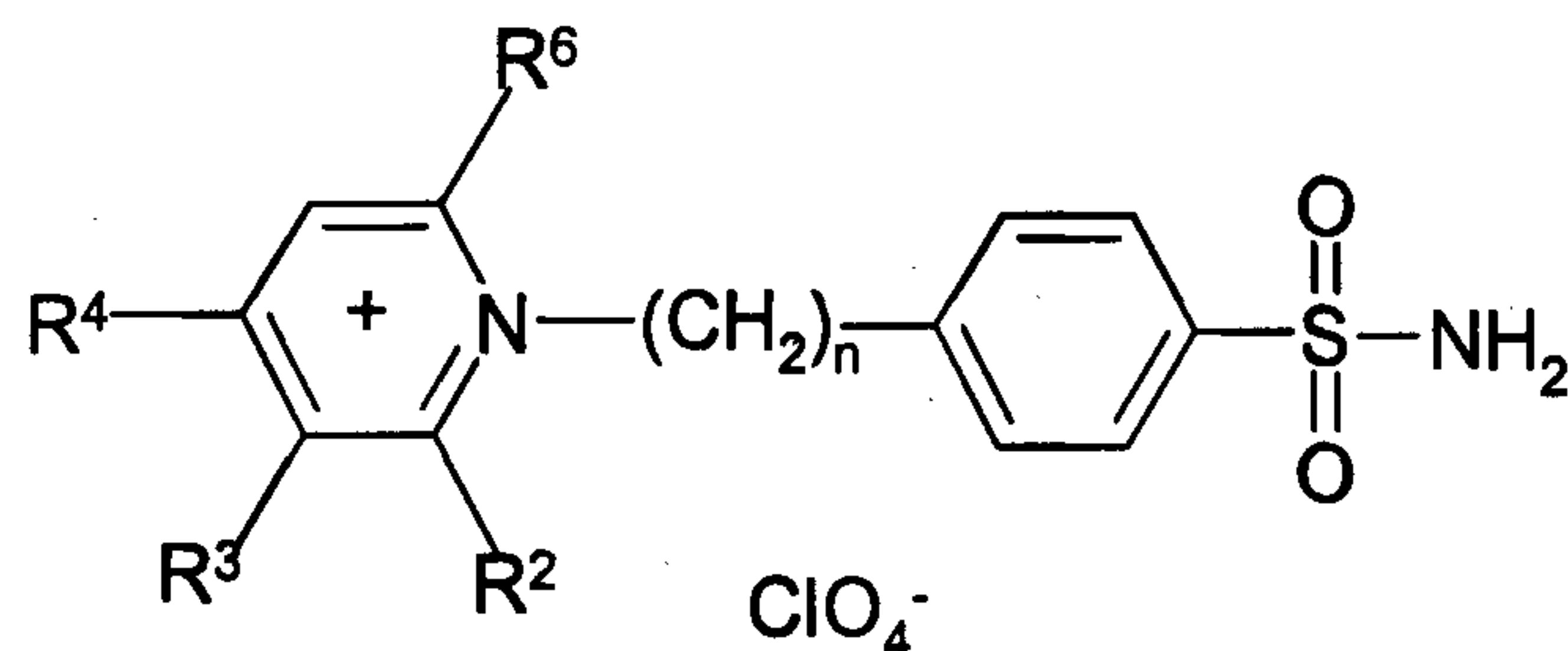


5. The method of claim 1, wherein said MN/CA IX-specific inhibitor is a membrane-impermeant, membrane impermeancy being determined by a membrane permeance assay, comprising the following steps:

- (a) incubating a millimolar solution of said MN/CA IX-specific inhibitor with human erythrocytes at 37°C for at least 30 minutes;
- (b) washing said human erythrocytes;
- (c) quantitating the levels of said MN/CA IX-specific inhibitor present in said human erythrocytes; and
- (d) if said MN/CA IX-specific inhibitor is present in said human erythrocytes at 1 micromolar or less, then said inhibitor is considered to be membrane-impermeant.

6. The method of claim 5, wherein said membrane-impermeant MN/CA IX-specific inhibitor is a pyridinium derivative of an aromatic sulfonamide or a pyridinium derivative of a heterocyclic sulfonamide.

7. The method of claim 6, wherein said pyridinium derivative of an aromatic sulfonamide has the general formula of:

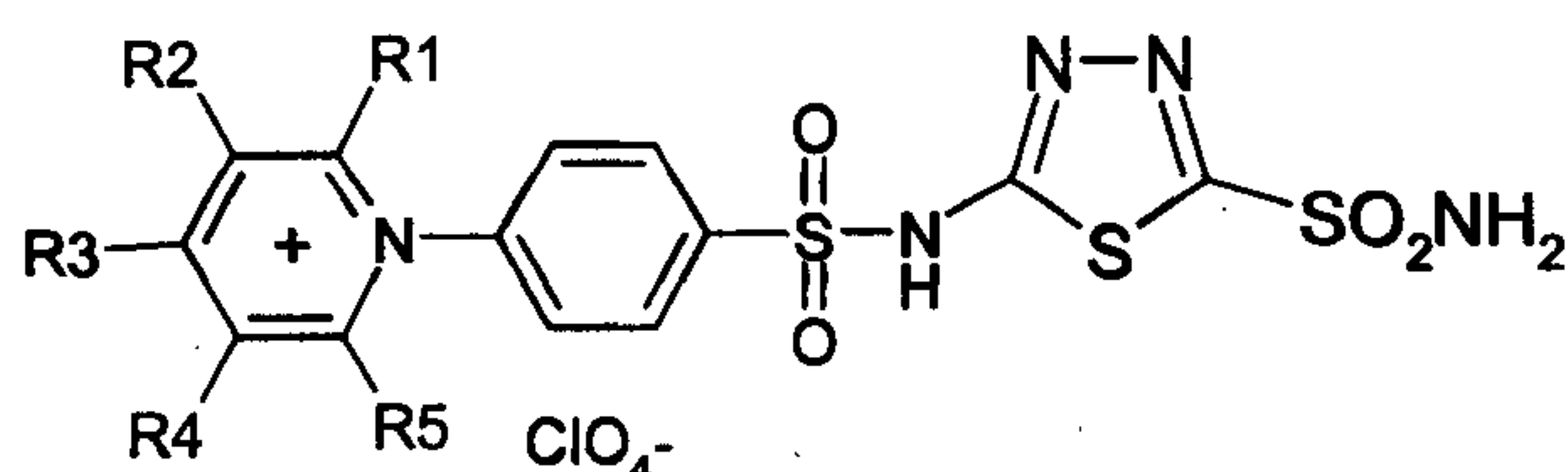


wherein

n is 0, 1, or 2;

R^2 , R^3 , R^4 and R^6 are each independently selected from the group consisting of hydrogen, alkyl moieties comprising from 1 to 12 carbon atoms, and aryl moieties.

8. The method of claim 6, wherein said pyridinium derivative of a heterocyclic sulfonamide has the general formula of:



wherein R^1 , R^2 , R^3 , R^4 and R^5 are each independently selected from the group consisting of hydrogen, alkyl moieties comprising from 1 to 12 carbon atoms, and aryl moieties.

9. A method for imaging tumors and metastases, or either tumors or metastases, that express MN/CA IX that is activated by hypoxic conditions in a

patient comprising the administration of a MN/CA IX-specific inhibitor linked to an imaging agent to said patient;

wherein said inhibitor is selected from the group consisting of organic heterocyclic and aromatic compounds, and wherein said inhibitor is a potent inhibitor of MN/CA IX enzymatic activity, wherein said inhibitor is considered to be such a potent inhibitor, if said inhibitor is tested in a screening assay comprising determining the inhibition constant K_I of said compound; and

if the inhibition constant K_I is less than 50 nanomolar, said inhibitor is considered to be a potent inhibitor of MN/CA IX enzymatic activity; and

wherein said potent inhibitor is considered to be an MN/CA IX-specific inhibitor, if it is a more potent inhibitor of MN/CA IX enzymatic activity than of the enzymatic activity of each of the carbonic anhydrases in a group consisting of CA I, CA II and CA IV.

10. The method of claim 9, wherein MN/CA IX activated by hypoxic conditions is detected or detected and quantitated, and the patient is considered to have a poorer prognosis than if said activated MN/CA IX is not detected, and decisions on treatment for said patient are made in view of the presence of said hypoxic conditions.

11. The method of claim 9, wherein said MN/CA IX-specific inhibitor is an aromatic sulfonamide or a heterocyclic sulfonamide.

12. The method of claim 9, wherein said MN/CA IX-specific inhibitor is a membrane-impermeant pyridinium derivative of an aromatic sulfonamide or a membrane-impermeant pyridinium derivative of a heterocyclic sulfonamide; wherein said compound is considered to be membrane-impermeant, if it is tested in a membrane permeance assay comprising the following steps:

- (a) incubating a millimolar solution of said MN/CA IX-specific inhibitor with human erythrocytes at 37°C for at least 30 minutes;
- (b) washing said human erythrocytes;

(c) quantitating the levels of said MN/CA IX-specific inhibitor present in said human erythrocytes; and

(d) if said MN/CA IX-specific inhibitor is present in said human erythrocytes at 1 micromolar or less, then said MN/CA IX-specific inhibitor is considered to be membrane-impermeant.

13. The use of a membrane-impermeant potent MN/CA IX-specific inhibitor in the treatment of a pre-cancerous or cancerous disease in a mammal, wherein said disease is characterized by overexpression of MN/CA IX protein, wherein said membrane-impermeant potent MN/CA IX-specific inhibitor is selected from the group consisting of organic heterocyclic and aromatic compounds;

wherein said MN/CA IX-specific inhibitor is considered to be membrane-impermeant if it is tested in a membrane permeance assay comprising:

(a) incubating a millimolar solution of said MN/CA IX-specific inhibitor with human erythrocytes at 37°C for at least 30 minutes;

(b) washing said human erythrocytes;

(c) quantitating the levels of said MN/CA IX-specific inhibitor present in said human erythrocytes; and

(d) if said MN/CA IX-specific inhibitor is present in said human erythrocytes at 1 micromolar or less, then said MN/CA IX-specific inhibitor is considered to be membrane-impermeant;

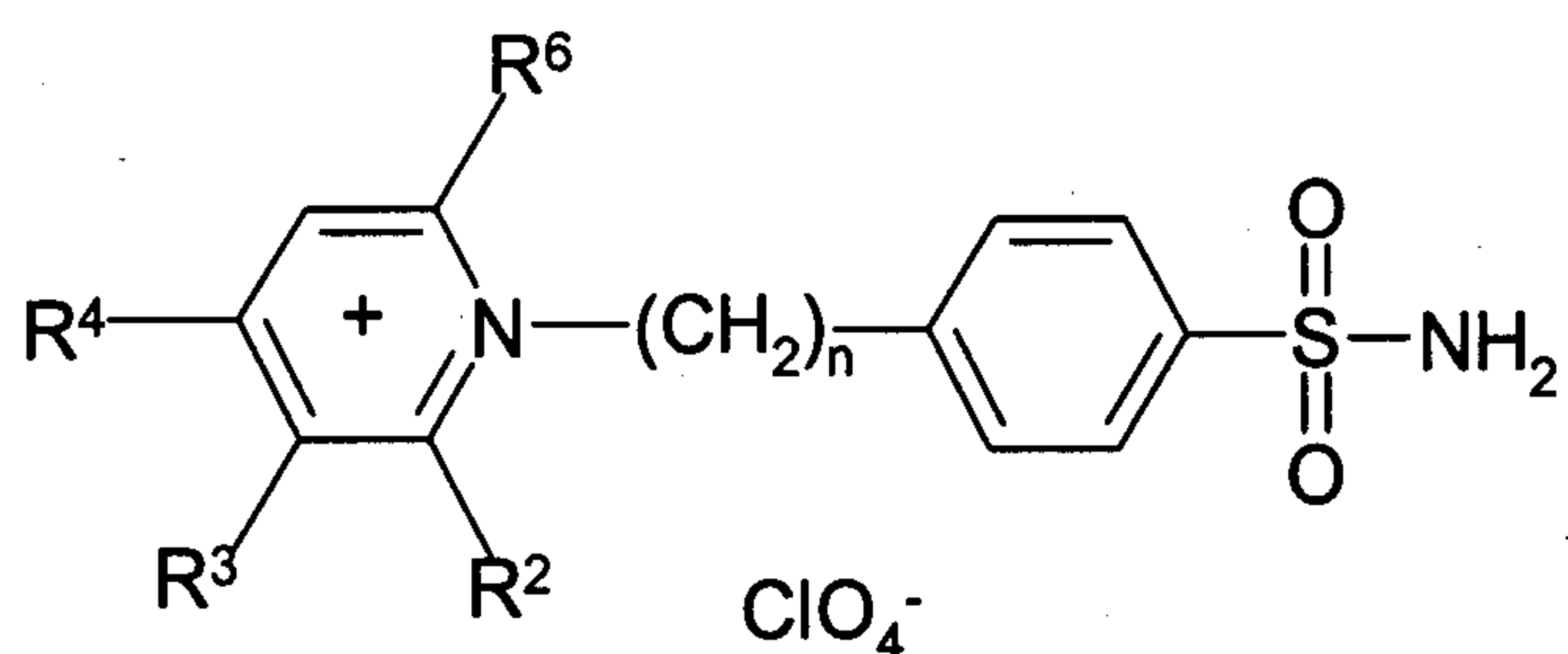
and wherein said membrane-impermeant MN/CA IX-specific inhibitor is considered to be a potent inhibitor of MN/CA IX enzymatic activity, if it is tested in a screening assay, comprising determining the inhibition constant K_i of said membrane-impermeant MN/CA IX-specific inhibitor,

wherein if the inhibition constant K_i is determined to be less than 50 nanomolar, said membrane-impermeant MN/CA IX-specific inhibitor is considered to be a potent inhibitor of MN/CA IX enzymatic activity.

14. The use as claimed in claim 13, wherein said membrane-impermeant potent MN/CA IX-specific inhibitor is a pyridinium derivative of an aromatic sulfonamide or a pyridinium derivative of a heterocyclic sulfonamide.

15. The use as claimed in claim 13, wherein said membrane-impermeant potent MN/CA IX-specific inhibitor is a more potent inhibitor of MN/CA IX enzymatic activity than of the enzymatic activity of CA IV.

16. The use as claimed in claim 14, wherein said pyridinium derivative of an aromatic sulfonamide has the general formula of:

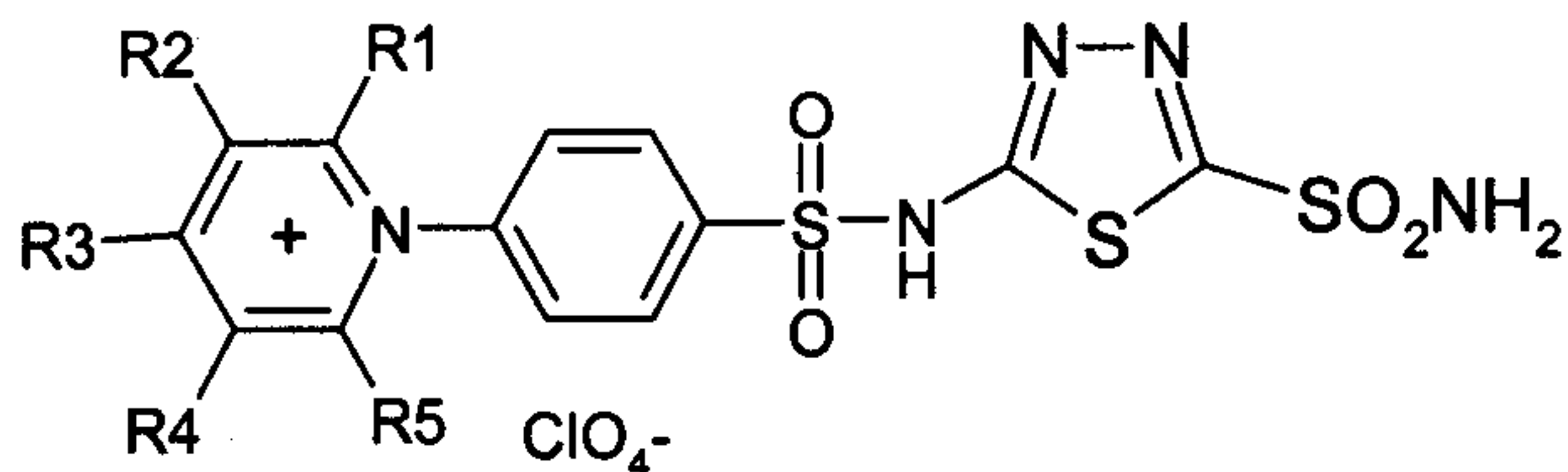


wherein

n is 0, 1, or 2;

R2, R3, R4 and R6 are each independently selected from the group consisting of hydrogen, alkyl moieties comprising from 1 to 12 carbon atoms, and aryl moieties.

17. The use as claimed in claim 14, wherein said pyridinium derivative of a heterocyclic sulfonamide has the general formula of:



wherein R1, R2, R3, R4 and R5 are each independently selected from the group consisting of hydrogen, alkyl moieties comprising from 1 to 12 carbon atoms, and aryl moieties.

18. The use of a compound to inhibit tumor growth in a patient having a tumor, the cells of which tumor are characterized by overexpression of MN/CA IX protein, wherein said compound is selected from the group consisting of organic heterocyclic and aromatic molecules, and wherein said compound is a potent and specific inhibitor of MN/CA IX enzymatic activity, wherein said inhibitor is considered to be such a potent inhibitor if said inhibitor is tested in a screening assay comprising determining the inhibition constant K_i of said compound, and if the inhibition constant K_i is less than 50 nanomolar, said compound is considered to be a potent inhibitor of MN/CA IX enzymatic activity;

wherein said potent inhibitor is considered to be an MN/CA IX-specific inhibitor, if it is a more potent inhibitor of MN/CA IX enzymatic activity than of the enzymatic activity of each of the carbonic anhydrases in a group consisting of CA I, CA II and CA IV;

and wherein said compound is not selected from the group consisting of acetazolamide, ethoxzolamide, methazolamide and cyanate.

19. The use as claimed in claim 18, wherein said potent MN/CA IX-specific inhibitor is membrane-impermeant, membrane impermeancy being determined by a membrane permeance assay, comprising the following steps:

- (a) incubating a millimolar solution of said potent MN/CA IX-specific inhibitor with human erythrocytes at 37°C for at least 30 minutes;
- (b) washing said human erythrocytes;
- (c) quantitating the levels of said potent MN/CA IX-specific inhibitor present in said human erythrocytes; and
- (d) if said potent MN/CA IX-specific inhibitor is present in said human erythrocytes at 1 micromolar or less, then said potent MN/CA IX-specific inhibitor is considered to be membrane-impermeant.

20. The use as claimed in claim 18, wherein said potent MN/CA IX-specific inhibitor is conjugated to a radioisotope before said use.

21. The use as claimed in claim 18 in conjunction with the use of radiation or a therapeutically effective amount in a physiologically acceptable formulation of one or more of the following compounds selected from the group consisting of: conventional anticancer drugs, chemotherapeutic agents, different inhibitors of cancer-related pathways, bioreductive drugs, MN/CA IX-specific antibodies and MN/CA IX-specific antibody fragments that are antigen-binding antibody fragments; or in conjunction with the use of both said radiation and said therapeutically effective amount of one or more compounds.

22. The use of a compound in the treatment of a pre-cancerous or cancerous disease in a mammal, wherein said disease is characterized by overexpression of MN/CA IX protein, wherein said compound is selected from the group consisting of organic heterocyclic and aromatic molecules, and wherein said compound is considered to be a potent and specific inhibitor of MN/CA IX enzymatic activity, wherein said inhibitor is considered to be such a potent inhibitor, if it is tested in a screening assay comprising determining the inhibition constant K_I of said compound, wherein said screening assay comprises:

- a) preparing serial dilutions of said compound and serial dilutions of MN/CA IX protein or a fragment of the MN/CA IX protein that comprises the carbonic anhydrase domain;
- b) preincubating a dilution of said compound with a dilution of said MN/CA IX protein or said MN/CA IX protein fragment for ten minutes at 20°C;
- c) combining said preincubated mixture of said diluted compound and said diluted MN/CA IX protein or protein fragment with a substrate, consisting essentially of a saturated CO₂ solution, phenol red to 0.2mM, Na₂SO₄ to 0.1M, and Hepes buffer (pH 7.5) to 10mM, in a reaction vessel for a period of 10 to 100 seconds at 20°C;

d) concurrently measuring the optical density, at the absorbance maximum of 557 nm, of the contents of said reaction vessel, using a stopped flow spectrophotometer; and

e) determining the inhibition constant K_i of said compound;

wherein if said inhibition constant K_i is less than 50 nanomolar, said compound is considered to be a potent inhibitor of MN/CA IX enzymatic activity; and

wherein said potent inhibitor is considered to be an MN/CA IX-specific inhibitor, if it is a more potent inhibitor of MN/CA IX enzymatic activity than of the enzymatic activity of each of the carbonic anhydrases in a group consisting of CA I, CA II and CA IV;

and wherein said compound is not selected from the group consisting of acetazolamide, ethoxzolamide, methazolamide and cyanate.

23. The use as claimed in claim 22, wherein said potent MN/CA IX-specific inhibitor is membrane-impermeant, membrane-impermeancy being determined by a membrane permeance assay comprising the following steps:

(a) incubating a millimolar solution of said potent MN/CA IX-specific inhibitor with human erythrocytes at 37°C for at least 30 minutes;

(b) washing said human erythrocytes;

(c) quantitating the levels of said potent MN/CA IX-specific inhibitor present in said human erythrocytes; and

(d) if said potent MN/CA IX-specific inhibitor is present in said human erythrocytes at 1 micromolar or less, said potent MN/CA IX-specific inhibitor is considered to be membrane-impermeant.

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173	F	C	P	A	L	R	P	L	E	L	L	G	F	Q	L	P	188
529	TTC	TGC	CCG	GCC	CTG	CTG	CCC	CTG	GAA	CTC	CTG	GGC	TTC	CAG	CTC	CCG	576
189	P	L	P	E	L	R	L	R	N	N	G	H	S	V	Q	L	204
577	CCG	CTC	CCA	GAA	CTG	CTG	CGC	CTG	AAC	AAT	GGC	CAC	AGT	GTG	CAA	CTG	624
205	T	L	P	P	G	L	E	M	A	L	G	P	G	R	E	Y	220
625	ACC	CTG	CCT	CCT	GGG	CTA	GAG	ATG	GCT	CTG	GGT	CCC	GGG	CGG	GAG	TAC	672
221	R	A	L	Q	L	H	L	H	W	G	A	A	G	R	P	G	236
673	CGG	GCT	CTG	CAG	CTG	CAT	CTG	CAC	TGG	GGG	GCT	GCA	GGT	CGT	CCG	GGC	720
237	S	E	H	T	V	E	G	H	R	F	P	A	E	I	H	V	252
721	TCG	GAG	CAC	ACT	GTG	GAA	GGC	CAC	CGT	TTC	CCT	GCC	GAG	ATC	CAC	GTG	768
253	V	H	L	S	T	A	F	A	R	V	D	E	A	L	G	R	268
769	GTT	CAC	CTC	AGC	ACC	ACC	GCC	TTT	GCC	AGA	GTT	GAC	GAG	GCC	TTG	GGG	816
269	P	G	G	L	A	V	L	A	A	F	L	E	E	G	P	E	284
817	CCG	GGA	GGC	CTG	GCC	GTG	TTG	GCC	GCC	TTT	CTG	GAG	GAG	GGC	CCG	GAA	864
285	E	N	S	A	Y	E	Q	L	L	S	R	L	E	E	I	A	300
865	GAA	AAC	AGT	GCC	TAT	GAG	CAG	TTG	CTG	TCT	CGC	TTG	GAA	GAA	ATC	GCT	912
301	E	E	G	S	E	T	Q	V	P	G	L	D	I	S	A	L	316
913	GAG	GAA	GGC	TCA	GAG	ACT	CAG	GTC	CCA	GGA	CTG	GAC	ATA	TCT	GCA	CTC	960
317	L	P	S	D	F	S	R	Y	F	Q	Y	E	G	S	L	T	332
961	CTG	CCC	TCT	GAC	TTC	AGC	CGC	TAC	TTC	CAA	TAT	GAG	GGG	TCT	CTG	ACT	1008
333	T	P	P	C	A	Q	G	V	I	W	T	V	F	N	Q	T	348
1009	ACA	CCG	CCC	TGT	GCC	CAG	GGT	GTC	ATC	TGG	ACT	GTG	TTT	AAC	CAG	ACA	1056

FIG.-1B

349 V M L S A K Q L H T L S D T L W 364
1057 GTG ATG CTG AGT GCT AAG CAG CAG CTC CAC CAC ACC CTC TCT GAC ACC CTG TGG 1104

365 G P G D S R L Q L N F R A T Q P 380
1105 GGA CCT GGT GAC TCT CGG CTA CAG CTG AAC TTC CGA GCG ACG CAG CCT 1152

381 L N G R V I E A S F P A G V D S 396
1153 TTG AAT GGG CGA GTG ATT GAG GCC TCC TTC CCT GCT GGA GTG GAC AGC 1200

397 S P R A A E P V Q L N S C L A A 412
1201 AGT CCT CGG GCT GCT GAG CCA GTC CAG CTG AAT TCC TGC CTG GCT GCT 1248

413 G D I L A L V F G L L F A V T S 428
1249 GGT GAC ATC CTA GCC CTG GTT TTT GGC CTC CTT TTT GCT GTC ACC AGC 1296

429 V A F L V Q M R R Q H R R G T K 444
1297 GTC GCG TTC CTT GTG CAG ATG AGA AGG CAG CAC AGA AGG GGA ACC AAA 1344

445 G G V S Y R P A E V A G A * 460
1345 GGG GGT GTG AGC TAC CGC CCA GCA GAG GTA GCC GAG ACT GGA GCC TAG 1392

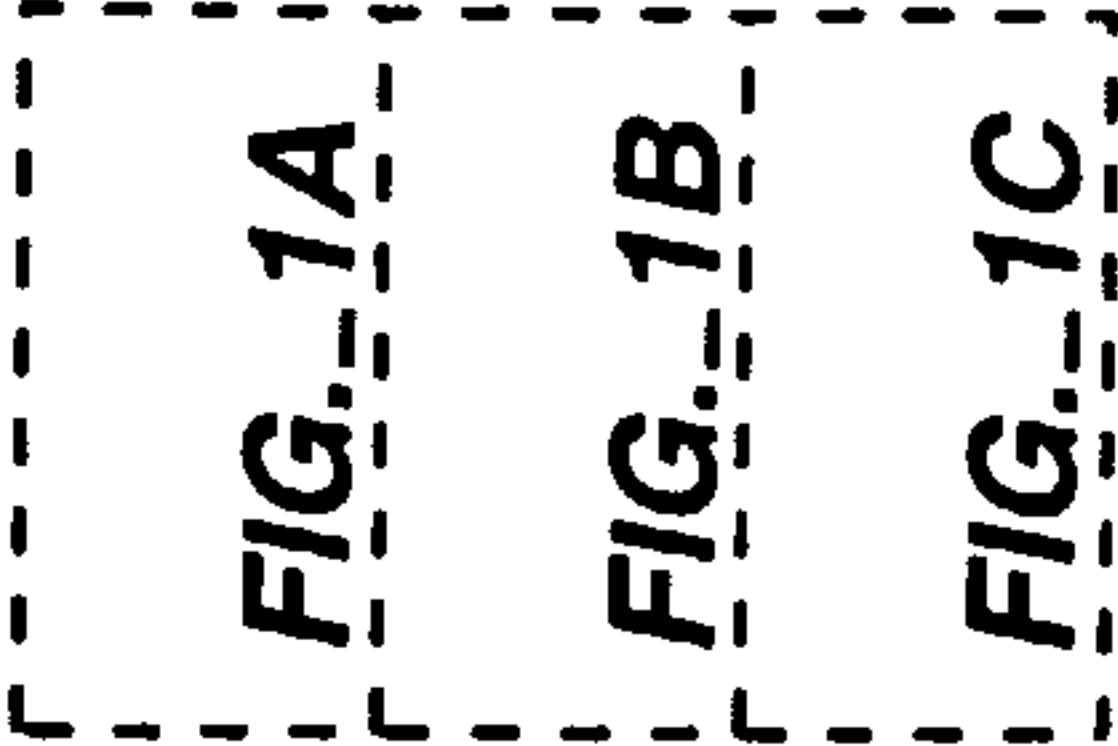
1393 AGG CTG GAT CTT GGA GAA TGT GAG AAG CCA GCC AGA GGC ATC TGA GGG 1440

1441 GGA GCC GGT AAC TGT CCT GTC CTG CTC ATT ATG CCA CTT CCT TTT AAC 1488

1489 TGC CAA GAA ATT TTT TAA AAT AAA TAT TTA TAA T 1522

FIG.-1C

FIG.-1



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1 ggatcctgtt gactcgtgac ctaccccca accctgtgct ctctgaaaca tgagctgtgt
61 ccactcaggg ttaaatggat taaggcggt gcaagatgtg ctttgttaaa cagatgcttg
121 aaggcagcat gctcgttaag agtcatcacc agtccctaat aatccctaata ctcaagtaat cagggacaca
181 aacactgcgg aaggccgcag ggtcctctgc ctatgtcca taaataaat taaataaaa aatacaaaa aaaaaaaa
241 ttatctgac ctccctcca ttatcaataa tagttattga taaatgaata atcacagctc aagtctacct gatttgatct
301 cacccaagaa ttatcaataa aaaaataaat tagttattga taaatgaata atcacagctc aagtctacct gatttgatct
361 aaaaaaaa gacttacgaa gacggccatc gacggccatc atcacagctc aagtctacct gatttgatct
421 aatgatcata ttcaaaacca gtcatctctt tacgttccaa acatttaggg gtaggaatga gcctttggct tatttttgta gctaattttg
481 ctctatcatt gtcatctctt tacgttccaa acatttaggg gtaggaatga gcctttggct tatttttgta gctaattttg
541 aagttctaat tacgttccaa acatttaggg gtaggaatga gcctttggct tatttttgta gctaattttg
601 ttgcttttga gccatgagtt acccttaagt atgcaactgt gtaggaatga gcctttggct tatttttgta gctaattttg
661 tttaaaactt acccttaagt atgcaactgt gtaggaatga gcctttggct tatttttgta gctaattttg
721 tagttaatgg atgcaactgt gtaggaatga gcctttggct tatttttgta gctaattttg
781 gggtaggtag gtactcagtt ttcaagtaatt gcttacctaa gaccctaagc cctatttctc
841 ttgtactggc ctttatctgt aatatgggca tatttaatac gatttaaat tggagtttt
901 tttgtttgtt tggtgcccac tggtgcccac tggtgcccac gatttaaat tggagtttt
961 ggagtagcag agcctcccga gtagctggga ctagggcctc gcaagctcca ctagggcctc gtagggcctc
1021 ttcctgcctc agcctcccga gtagctggga ctagggcctc gcaagctcca ctagggcctc gtagggcctc
1081 ttttttgtat ttttggtaga gacggggttt gagtctttta aagtaaaaat atgtcttgta agctggtaac
1141 ctgacttcgt gatccaccg cctcggcctc gagtctttta aagtaaaaat atgtcttgta agctggtaac
1201 ccgcacctgg ccaattttt cactcctttt taatgtgtg ctgacgggtca ttaggttct tttgagtttg
1261 tatggtacat ttccttttat gctactttt gcagtcctt cactacatt cattacatt tctctcttc attgaagag
1321 gcatgcatat gctactttt cactttagctt cacttggctt aaaaaggctt gaaaacacgt caagaaattg cacagtaata
1381 catgttatat cttttagctt taccacttgg agagggatga ttcagggtgaa ggttttctt tttttccag agagaggtct
1441 tcattgttgg taccacttgg agagggatga ttcagggtgaa ggttttctt tttttccag agagaggtct
1501 cttgtttgta agagggatga ttcagggtgaa ggttttctt tttttccag agagaggtct
1561 tctgagattc ctctgacatt gctgtatata ggttttctt tttttccag agagaggtct
1621 actattttt ttaagcaaga tatgctaaag ttttgtgagc tttttccag agagaggtct
1681 catatctgca tcaagtgaga acatatatg tctgcatgtt tccatatctt cctcagtgac ccaaaagag
1741 gcttgtgttt tatgctttta tatagacagg gaaacttgtt cctcagtgac ccaaaagag
1801 tgggaattgt tattggatat catcatctgg ccacgcttcc tacccttggg aacaattaa
1861 ggttcataat ctcaattctg tcagaattgg tacaagaaat tgacgttatg agctgctatg tttcttgaca
1921 tccacttgg taggaaataa gaatgtgaaa ctcttcagtt ggtgtgtgtc cct?gtttt

FIG._2A

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1981 ttgcaatttc cttcttactg tgtaaataa aagtatgac ttgctctgag aggtgaggca
2041 ttcttaatca tgatctttaa agatcaataa tataatcctt tcaaggatta tgtctttatt
2101 ataataaaga taatttgtct gcagtggctc acacctgtaa tcccagcact atcccttaaa ggattatatc
2161 ttgctgggc gatcaaat ttgacttctt atattatctt acacctgtaa tgggtggcc aaggtggaag
2221 tatgatgata ttgacagggt ttgacctcac ttgacctcac ttaagcaga attcatctct ctccctcaa
2281 ggtagcgttt ttgttttttg ttgttttttg ttgttttttg tctttttga gacagggtct gctcaggga
2341 cccaggccag agtgcaatgg tacagtctca gctcactgca gctcaaccg gcctcaaccg cctcggctca
2401 aaccatcatc ttttgtatct ccatctcagc ctctgagta gctgggacta caggcacatg ccattacacc
2461 tggctaattt ttttgtatct ccatctcagc ctctgagta gctgggacta caggcacatg ccattacacc
2521 tcgaactcct ggactcaagc aatccacca aatccacca cctcagcctc ccaaatgag ggaccgtgtc
2581 ttattcatct ccatgtccct gaatgcaata gtattggaga gtttaatttg gctagagtat gagggagagt
2701 aatatttgtt gtggtaaaag agtaggagac gtgcataatc ttgaaaaata aatcaagctc cttcaccctc
2761 gtggtaaaag gtattggaga gttttggaga gttttggaga gttttggaga gttttggaga gttttggaga
2821 agtaggagac agtaggagac agtaggagac agtaggagac agtaggagac agtaggagac agtaggagac
2881 agtaggagac agtaggagac agtaggagac agtaggagac agtaggagac agtaggagac agtaggagac
2941 gagtaatgtg ttgaaaaata attcaagctc attcaagctc attcaagctc attcaagctc attcaagctc
3001 cttgcttttc ggctccccta ggctccccta ggctccccta ggctccccta ggctccccta ggctccccta
3061 ggctccccta ggctccccta ggctccccta ggctccccta ggctccccta ggctccccta ggctccccta
3121 acatgagctg ctttccctct ctttccctct ctttccctct ctttccctct ctttccctct ctttccctct
3181 cccttctgtg cctggagctg cctggagctg cctggagctg cctggagctg cctggagctg cctggagctg
3241 ctgggtggtg ccaggagagag ccaggagagag ccaggagagag ccaggagagag ccaggagagag ccaggagagag
3301 ccaggagagag ccaggagagag ccaggagagag ccaggagagag ccaggagagag ccaggagagag ccaggagagag
3361 agctttggta tgggggagag tgggggagag tgggggagag tgggggagag tgggggagag tgggggagag
3421 tctgcaaaag ggcgctctgt ggcgctctgt ggcgctctgt ggcgctctgt ggcgctctgt ggcgctctgt
3481 cagctctcgt ttccaatgca ttccaatgca ttccaatgca ttccaatgca ttccaatgca ttccaatgca
3541 TCAGCCGCAT GGCTCCCCCTG GGCTCCCCCTG GGCTCCCCCTG GGCTCCCCCTG GGCTCCCCCTG GGCTCCCCCTG
3601 CTCAGGCCT CACTGTGCAA CACTGTGCAA CACTGTGCAA CACTGTGCAA CACTGTGCAA CACTGTGCAA
3661 AGAGGTTGCC CCGGATGCAG CCGGATGCAG CCGGATGCAG CCGGATGCAG CCGGATGCAG CCGGATGCAG
3721 ACCCACTGGG CGAGGAGGAT CGAGGAGGAT CGAGGAGGAT CGAGGAGGAT CGAGGAGGAT CGAGGAGGAT
3781 CCGGAGAGGA GGATCTACCT GGATCTACCT GGATCTACCT GGATCTACCT GGATCTACCT GGATCTACCT
3841 TTAAGCCTAA ATCAGAAGAA ATCAGAAGAA ATCAGAAGAA ATCAGAAGAA ATCAGAAGAA ATCAGAAGAA
3901 CTCCTGGAGA TCCTCAAGAA TCCTCAAGAA TCCTCAAGAA TCCTCAAGAA TCCTCAAGAA TCCTCAAGAA

FIG.-2B

3961 catcaatctc caaatccagg ttccaggagg ttcatgactc ccctcccata cccagccta
4021 ggctctgttc actcagggaa ggaggggaga ctgtactccc cacagaagcc ctccagagg
4081 tcccatacca atatcccat cccactctc ggaggtagaa aggacagat gtgagagaa
4141 aataaaaagg gtgcaaaaagg agagaggtaga gctggatgag atgggagaga aggggaggc
4201 tggagaagag aaagggatga gaactgcaga tgagagaaa tgagagagaa cagaggaaaa
4261 aaataggtag gtgaagtggg taccagagac aagcaagaag tgaggggaag agaaaaggaa agcttgggag
4321 gtgaagtggg caatgaggaa ttgagacctt ggaagaagg acacagcagg tagagaaacg tggcttcttg
4381 caatgaggaa actccaagc caggaaatttg ggaagaagg ttggagacca ttacaaggcag aggatgagt
4441 actccaagc ggggagaaga tgaagtggc actcactttt cgaatctggc ttagctactc actcatttg gactcaggac
4501 ggggagaaga tgaagtggc caggaatttg aagaaggag aaaggaaaga tgggtgactc tttttgagac
4561 tgaagtggc caggaatttg aagaaggag aaaggaaaga tttttttttt tttttgagac aaactttcac
4621 caggctggag tgcaatggcg gcctcagcct ttgtatattt atctcaggtg atccaaccac cctggcctcc
4681 tgattctcct cgggctaatt cgaactcctg cgtgagccac ttgcaagctg gtaggattgc tggttgagc
4741 cgggctaatt cgaactcctg cgtgagccac ttgcaagctg gtaggattgc tggttgagc
4801 cgaactcctg cgtgagccac ttgcaagctg gtaggattgc tggttgagc
4861 cgtgagccac ttgcaagctg gtaggattgc tggttgagc
4921 ttgcaagctg gtaggattgc tggttgagc
4981 tctcctgtgc gcatctgcgt ttgtgacatc ttttcattta ccaatctgg gggcgtccca
5041 gcatctgcgt ttgtgacatc ttttcattta ccaatctgg gggcgtccca
5101 cggttcatcc acacccaccc cgtccctgaa tttctaccg caccaccaggc
5161 acacccaccc cgtccctgaa tttctaccg caccaccaggc
5221 cgtccctgaa tttctaccg caccaccaggc
5281 tttctaccg caccaccaggc
5341 caccaccaggc
5401 CCCGGTGGAT CCTGGGCTTC
5461 CCTGGGCTTC
5521 tgaggggggtc
5581 cgcagtgcct
5641 ccctacgcag
5701 GAGTACCGGG
5761 CACACTGTGG
5821 aaaggagcgg
5881 agATCCACGT

cccagccta
ctccagagg
gtgagagaa
aggggaggc
cagaggaaaa
agcttgggag
tcttaggcta
tggcttcttg
aggatgagt
gactcaggac
tttgttgcc
cgggttcaag
gccaccacgc
aggctggtct
ggattatagg
aagacaatga
ttgggtgcgg
ctcctgtaag
taagcttgag
TGGAGgtgag
cccctacagc
cccctcacct
tatactctcc
GCTTCCAGTC
CCTGGAACT
GCCACAGTGg
ggaaccgtcg
acttgccctc
TCCCGGGCGG
GGCTCGGAG
gagaaggggc
tgtcctttc
TTGGGGCGCC

ccctcccata
cacagaagcc
aggacagat
atgggagaga
aatgtgcaga
agaaaaggaa
agtcactctca
tagagaaacg
tacaaggcag
actcatttg
aaactttcac
ctccacctcc
acaggcatgc
catgttggtc
caaagtgcctg
tacagaccct
gggttgagc
accgtaatg
attggggctc
ATTGGCGCTA
ctgtggatct
accgtcccac
acttctctca
TGCGCGGGCC
GCCCTGCGCC
CGCAACAATG
cgcagggaag
gggccggctc
TGGCTCTGGG
CAGGTCGTCC
cggactggcc
cctaccctcg
TGACGAGGCC

ttcatgactc
ctgtactccc
ggaggtagaa
gctggatgag
tgagagaaa
tgaggggaag
agctggtaga
acacagcagg
ttggagacca
tggtgtactc
tttttgagac
tactgcaac
agctgcgatt
gggtttcgc
cctggcctcc
cactcacttt
accagctgc
ggcatttgtt
ccaggaagg
GACCAGAGTC
gaaccagct
cccgcgccc
taggcgtcag
GTCCCCAGCC
CTTCTGCCCG
ACTGCGCCTG
tggggcgggg
ctaccgggcg
GGCTAGAGA
TGGGGGCTG
GAGgtgagcg
gtggccctct
TTGCCAGAGT

ttccaggagg
ggaggggaga
cccactctc
agagaggtaga
gaactgcaga
tcagagagtt
aagcaagaag
ggaagaagg
gggaagaagg
aaaggaaaga
tttttttttt
cgaatctggc
ctagccaagt
tagtagagac
atccaaccac
ctgaagcagc
tgtttggccc
gcccgccttaa
gttttggtcg
tacagGGGAT
ccaatctgg
ggcgtccca
gttcctgacc
GGCCCCGGGT
AGCTCGCCGC
CGCTCCCAGA
gacttggga
tggtctggcc
CCTGCCCTCCT
GCATCTGCAC
TTTCCCTGCC
ggccagagac
AGCACCGCCT

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5941 CGGGAGGCCT GCGGTGTTG GCGCCTTTC TGGAGgtacc agatcctgga cacccttac
6001 tcccgcctt ccatcccat gctcctccc gactctatcg tggagccaga gaccccatcc
6061 cagcaagctc actcaggccc ctggctgaca aactcattca cgcactgttt gtcatttaa
6121 caccactgt gaaccaggca ccagccccc tgggggaggc tgaagctgta ggtccttgcc
6181 tctaaggagc ccacagccag ggtcacagag tgacatgaca gacacatagg aaggacatag
6241 taaagatggt ggtcacagag gaggtgacac ttaaagcctt cactggtaga aaagaaaagg
6301 aggtgttcat tgcagaggaa acagaatgtg caaagactca gaatatggcc tatttaggga
6361 atggctacat acaccatgat tagaggaggc ccagttaaagg gaagggatgg tgagatgcct
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6481 ccaggctgga gtgcagtgggt gtgatcttgg gtcactgcaa ctccgcctc ccgggttcaa
6541 gggattctcc tgcctcagct tcctgagtag ctgggggttac agtgtgtgtc caccatgccc
6601 agctaatttt tttttgtatt tttagtagac aggttttcac catgttggtc aggctggtct
6661 caaactcctg gcctcaagtg atccgcctga ctcagcctac ccaagtgtg attacaagtg
6721 tgagccaccg tgcccagcca cactcactga ttctttaatg ccagccacac agcacaagt
6781 tcagagaaat gcctccatca tagcatgtca atatgttcat actcttaggt tcatgatgtt
6841 cttaacatta ggttcataag caaataaga aaaaagaata ataataaaa gaagtggcat
6901 gtcaggacct cacctgaaaa cccaaacaca gaatcatgaa ggtgaatgca gagtgacac
6961 caacacaaag gtgtatatat ggtttcctgt ggggagtatg tacggaggca gcagtgagtg
7021 agactgcaaa cgtcagaagg gcacgggtca ctgagagcct agtatcctag taaagtgggc
7081 tctctccctc tctctccagc ttgtcattga aaaccagtcc accaagcttg ttggttcgca
7141 cagcaagagt acatagagt tgaataata cataaggatt taagagggag aactgtcttc
7201 taataaaaaa aacaacagca acaacaaaaa gcaacaacca ttacaatttt atgttccctc
7261 agcatcttca gagctgagga atgggagagg actatgggaa ccccttcat gtccggcct
7321 tcagccatgg ccctggatac atgcactcat ctgtcttaca atgtcattcc ccagGAGGG
7381 CCCGGAAGAA AACAGTGCC TCGCTGAGGA TTGGAAGAAA
7441 AGgtcagttt gttggtctgg ccactaatct ctgtggccta gtcataaag aatcacctt
7501 tggagcttca ggtctgaggc tggagatggg ctccctccag tgcaggaggg attgaagcat
7561 gagccagcgc tcatcttgat aataaccatg aagctgacag acacagttac ccgcaaacgg
7621 ctgacctacg attgaaaacc aagcaaaaac cgccgggcac ggtggctcac gcctgtaac
7681 ccagcacttt gggaggccaa ggcagggtga tcacgaggtc aagagatcaa gaccatcctg
7741 gccaacatgg tgaaacccca tcttactaa aaatagccag aaatagccag gcgtggtggc
7801 gggtgccctgt aatcccagct actcgggagg ctgaggcagg agaattggcat gaaccggga
7861 ggcagaaagt gcaagtgagcc gagatcgtgc cactgcactc cagcctgggc aacagagcga

FIG. 2D

7921 gactcttgct tcaaaaaaa aaaaaaaa gaaaaccaag caaaaccaa aatgagacaa
7981 aaaaaacaag accaaaaaat ggtgtttgga aatgttcaag gtcaagtcctg gagagctaaa
8041 ctctttctga gaactgttta tctttaataa gcatcaataa ctttaacttt gtaaatactt
8101 ttgttggaat ttgttctctt cttagtcact cttagtgctat tttaaatctc acttactcta
8161 ctgaccttt taggtttctg cttagactagg cttagactctg tagaactctg ctttgcatc tcttggtctt
8221 gttttgtata gttatcaata ttcatattta ttacaagt tttagtaga attcagatca ttttttctt
8281 tctttttttt tttttttttt tttttttacat cttagtaga gacagggtt ccaagtgct caccatattg
8341 gccaggctgc tctcaaaact tttcctttt tttcctttgt gatccaccag cctcggcctc caaagtgct
8401 gggattcatt ttttctttt aatttgctct aatttgctct gggcttaaac ttgtggcca gacttttatg
8461 atggtacaca ggttaagag gagttaagag tgtagactca gacggtcttt cttctttcct tctcttcct
8521 cctcccttcc caggttgctc caagccctg ccaagccctg tactttttt tcttccctc ttgcttcct
8581 caggtcctct agggcctgca cttagtgaag aagtggctct aagtgtgagt aggttaacg tcttatggga
8641 gaaactgtat tagatcctct tcacagGCTC AGAGACTCAG GTCCAGGAC GTCCAGTATC TGCACTCCTG
8701 tagatcctct CCTCTGACT TCAGCCGCTA CTTCATAAT GAGGGTCTC ACAGTGATGC TGAGTGCTAA GCAGgtggc
8761 CAGGGTGTCA TCTGGACTGT TGTGGACACA GTGGgtgcgg ggtgaagag atgtaagatg agatgagaaa
8821 ctgggggtgtg aggagaaga caggagaaga ggtgggagaa ggtgggcttc tggtggcttac gcctataatc ccaccacgtt
8881 ggtgggctga agtgtgacc catctctacc tagtcccagc ctatgatccc aagaggctgg atggggaata caggagctgg aggtggagc
8941 agtgtgacc gtatgcggcc gagtttgaga gagtttttat atttatatat cctgaggctg tggttgtgag acccttggtt accgaacca
9001 caggagaaga caggagaaga ggtgggagaa catctctacc tagtcccagc ctatgatccc aagaggctgg atggggaata caggagctgg aggtggagc
9061 ggtgggctga agtgtgacc catctctacc tagtcccagc ctatgatccc aagaggctgg atggggaata caggagctgg aggtggagc
9121 agtgtgacc gtatgcggcc gagtttgaga gagtttttat atttatatat cctgaggctg tggttgtgag acccttggtt accgaacca
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9241 gagtttgaga gagtttttat atttatatat cctgaggctg tggttgtgag acccttggtt accgaacca
9301 atttatatat cctgaggctg tggttgtgag gagtttttat accatcttta caggagctgg caggagctgg cctgtcatgc TGACACCTTG
9361 cctgaggctg tggttgtgag gagtttttat accatcttta caggagctgg caggagctgg cctgtcatgc TGACACCTTG
9421 cccacactgt GTGACTCTCG GCTACAGCTG AACTTCCGAG CGACGCAGCC GTCCCTCGGC TGCTGAGCCA Ggtacagctt
9481 GTGACTCTCG GCTACAGCTG AACTTCCGAG CGACGCAGCC GTCCCTCGGC TGCTGAGCCA Ggtacagctt
9541 AGGCCTCCTT CCCTGCTGGA GTGGACAGCA GTCCCTCGGC TGCTGAGCCA Ggtacagctt
9601 tgtctgggtt ccccccagcc agtagtccct agtagtccca tgtgtgtgcc agtgtctgtc
9661 attgggtggc acagcccgcg tctcacatct cctttttctc tccagTCCAG CTGAATTCTT
9721 GCCTGGCTGC TGgtgagtct gcccctcctc ttggtcctga tggcaggaga ctcctcagca
9781 ccattcagcc ccagggtgc tcaggaccgc cctgtctccc ctctcctttc tgcagaacag
9841 accccaacc caatattaga gaggcagatc atggtgggga tcccccat tccccagag

FIG.-2E

9901 gctaattgat tagaatgaag cttgagaaat ctcccagcat ccctctcgca aaagaatccc
9961 cccccctttt tttaagata gggctctcact ctgtttgccc caggctgggg tgttgtggca
10021 cgatcatagc tcaatgcagc tgggactgta gcctcttctg gagactgagg cactatgggg ctgcctgaga actcggggca
10081 ctcaaagcac ttgggcttta ggaagcaaaa acggtgctta tcttaccctt actgtgcctg gcccacaacg gccctttttac
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10261 ggggtggctg ggggtggctg atgcaaatga gctgctcctg gctgctcctt acccttgtgg agtcacttca tgcaaaagcgc
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10441 tgacccttcc atgcaaatga gctgctcctg gctgctcctt gggcagtttt ctgattagcc ttccctgttg tgtacacaca
10501 atgcaaatga gctgctcctg gctgctcctt gggcagtttt ctgattagcc ttccctgttg tgtacacaca
10561 gAAGGGGAAc CAAAGGGGGT GTGAGCTACC GCCCAGCAGA GGTAGCCGAG ACTGGAGCCTT
10621 AGAGGCTGGA TCTTGGAGAA TGTGAGAAGC CAGCCAGAGG CATCTGAGGG GGAGCCGGTA
10681 ACTGTCCTGT CCTGCTCATT ATGCCACTTC CTTTAACTG CCAAGAAATT TTTTAAATA
10741 AATAATTATA ATaaatatg tgtagtcac ctttggtccc caaatcagaa ggaggtattt
10801 gaatttccta ttactgttat tagcaccaat ttagtggtaa tgcatttatt ctattacagt
10861 tcggcctcct tccacacatc actccaatgt gttgctcc

FIG.-2F

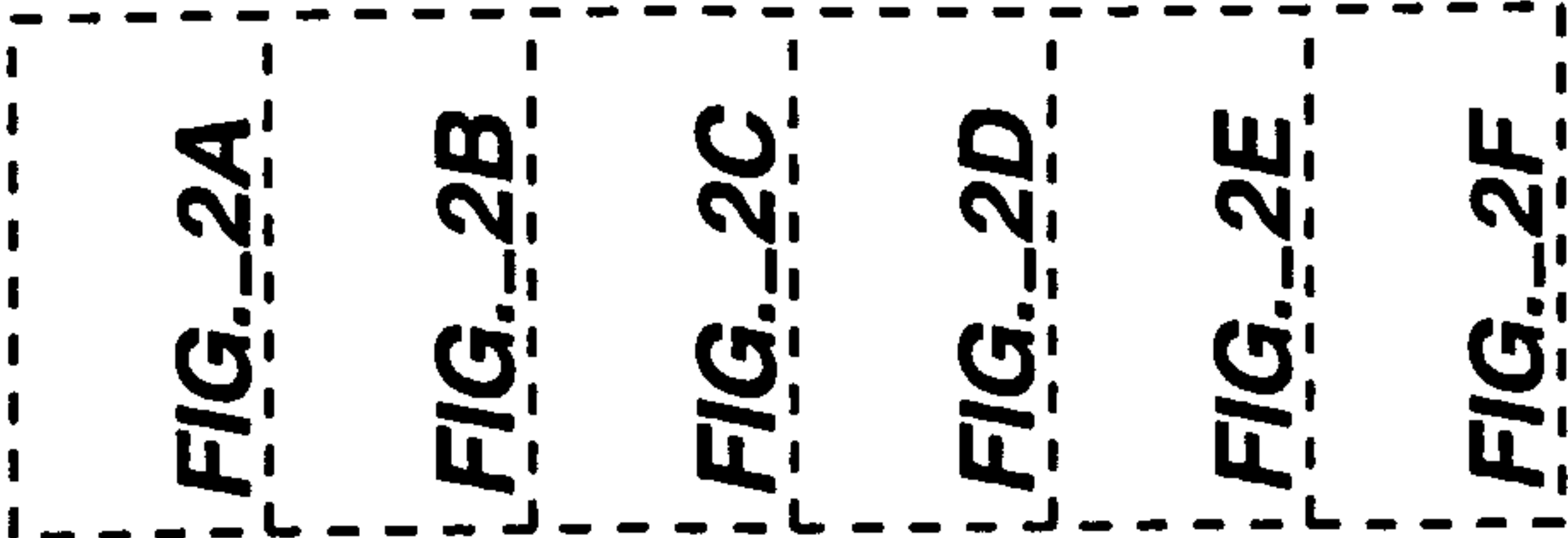


FIG.-2

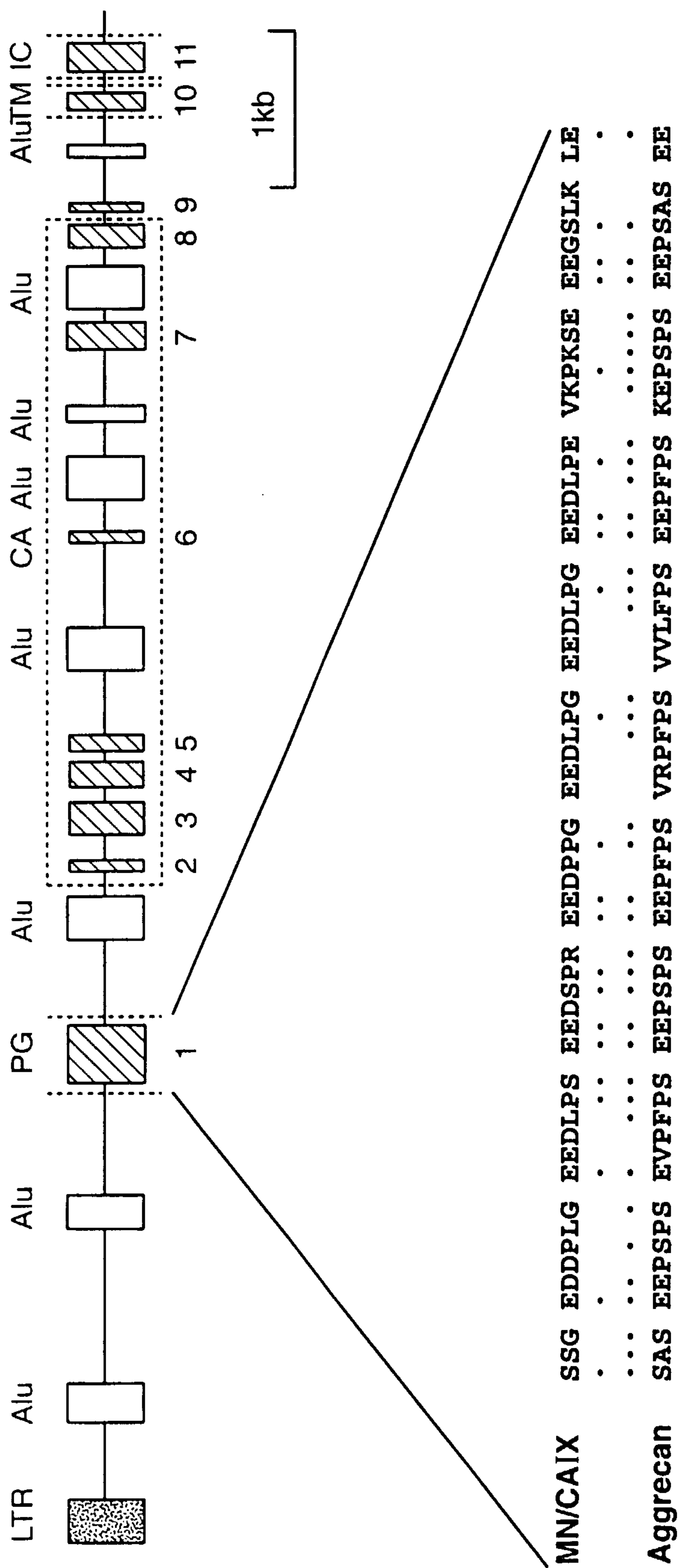
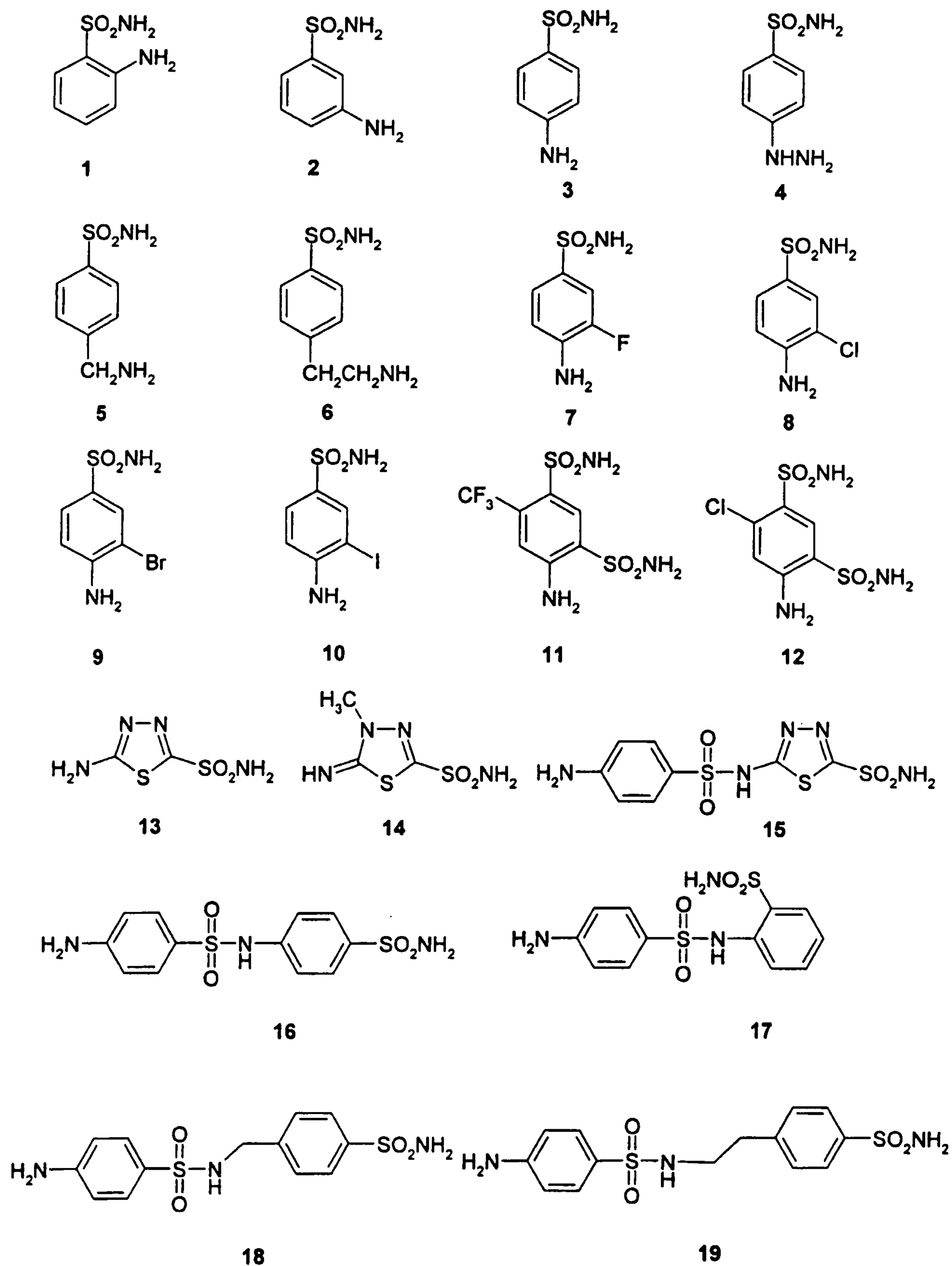
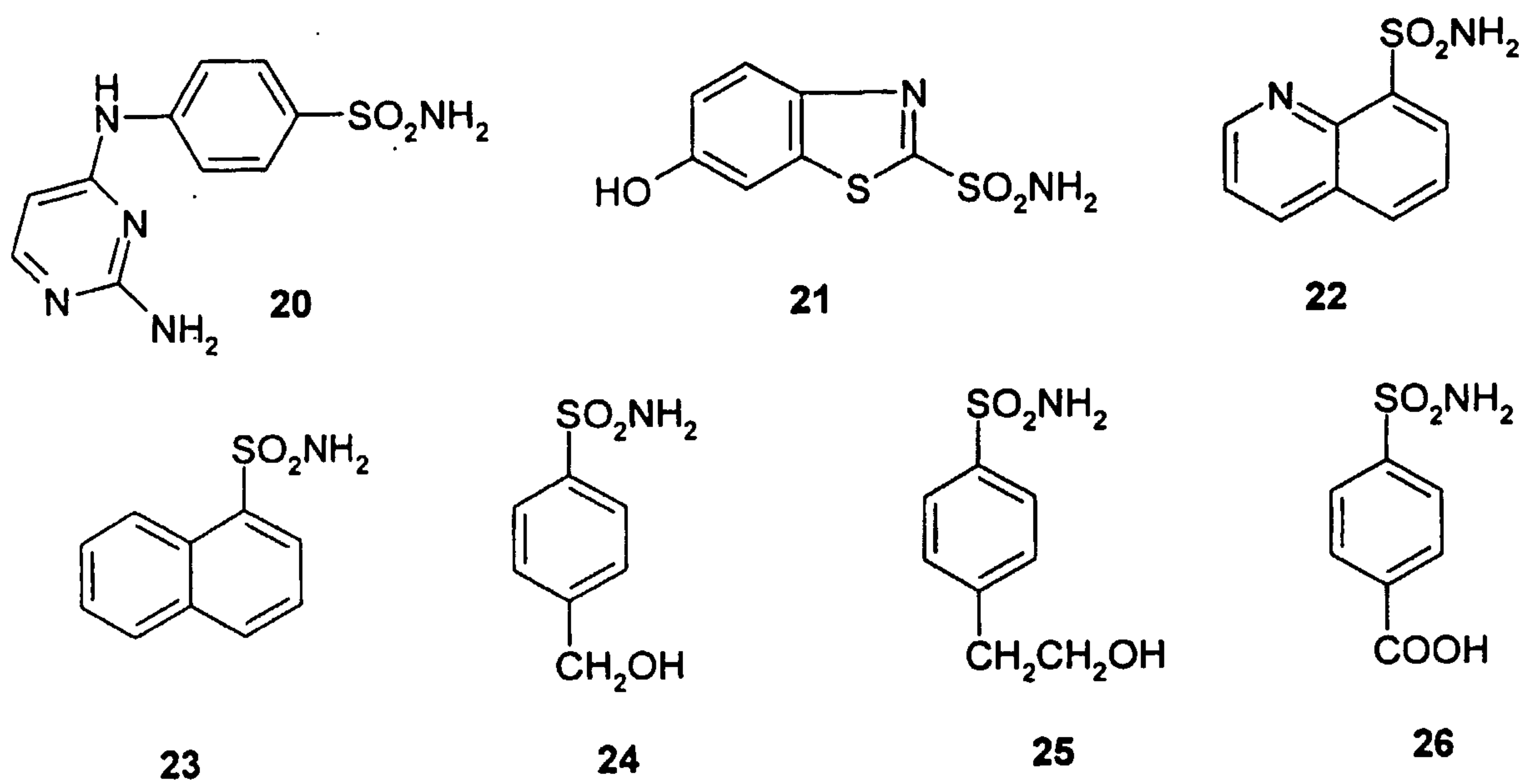


FIG. 3

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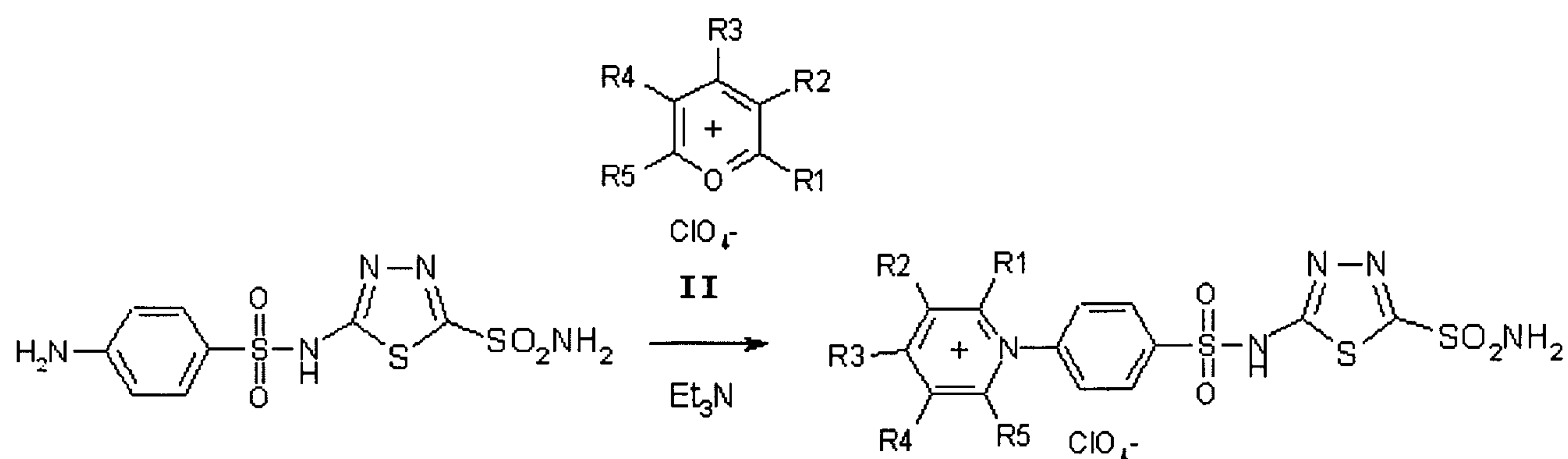
**FIG. 4A**

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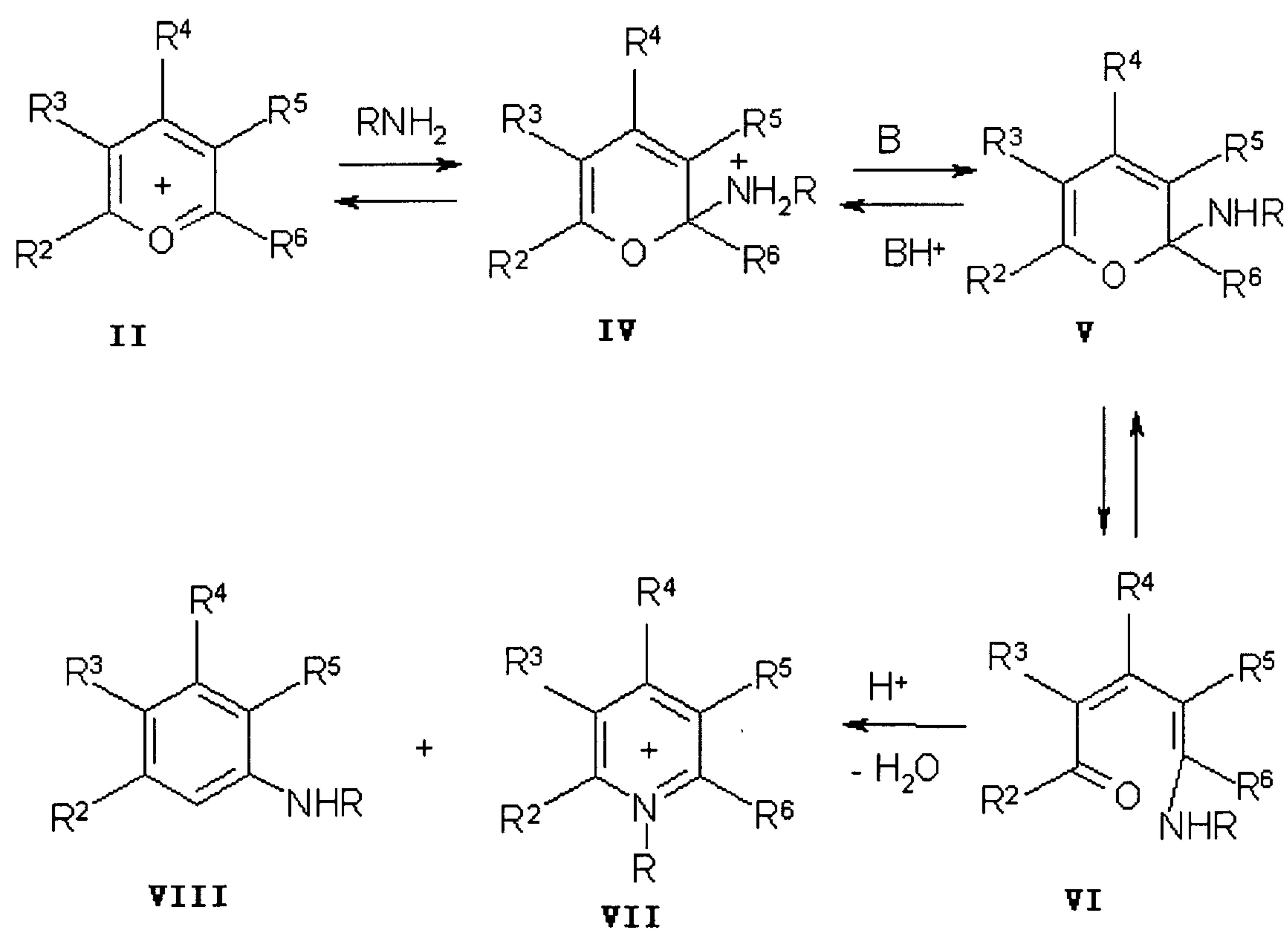
**FIG._4B**

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Scheme 1

**FIG._5**

Scheme 2

**FIG._6**