Carbonic Anhydrase Inhibitors: The First On-Resin Screening of a 4-Sulfamoylphenylthiourea Library

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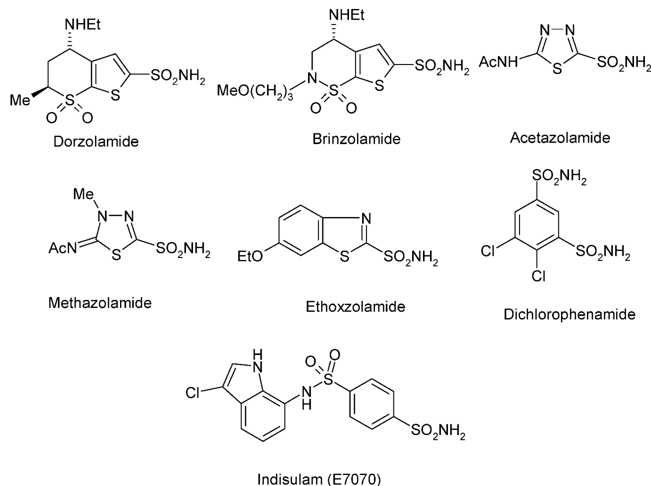
Sulfonamide carbonic anhydrase (CA) inhibitors are widely employed in the diagnosis and treatment of diverse diseases such as glaucoma and different neuromuscular disorders. Moreover, an emerging area is represented by their use in the prevention and treatment of tumors. In this paper we propose an optimized synthesis of on-resin CA inhibitor libraries to be used for a high-throughput biological screening. A library of 4-sulfamoylphenylthioureas, previously described to be attractive candidates as novel antiglaucoma drugs, has been synthesized by a solid-phase approach, avoiding the formation of thiohydantoin side products. The on-resin screening assay has been developed for the inhibition tests of different CA isozymes with the on-resin supported sulfonamides, allowing the direct identification of the biologically active lead compounds. These results allow the development of new designed libraries in the solid phase of sulfonamide CA inhibitors characterized by a set of prefixed parameters to be used as possible drug candidates.

Introduction

Sulfonamide inhibitors of the metalloenzyme carbonic anhydrase (CA, EC 4.2.1.1) are extensively used in clinical medicine, and as diagnostic tools for the treatment of glaucoma and macular edema, diverse neuromuscular disorders, or as antitumor drugs.[1−5] Some of these clinically used drugs include the topically acting antiglaucoma agents dorzolamide and brinzolamide, the systemic inhibitors acetazolamide, methazolamide, ethoxzolamide, and dichlorophenamide, whereas indisulam (E7070) is in phase II clinical trials as an antitumor sulfonamide with a complex mechanism of action also involving CA inhibition of several isozymes participating in tumor genesis (Chart 1).[4,5]

Chart 1



As mentioned above, most of these compounds 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).[1,2] Therefore, many attempts to design and synthesize new sulfonamides were recently reported, in order to avoid such side effects.[6−10] The most effective synthetic approach consisted in attaching hydrophilic "tails" to a scaffold of aromatic/heterocyclic sulfonamides, possessing amino, imino, or hydroxyl moieties in their molecules.[6−9] The presence of such moieties had as the main objective the possibility to formulate these CA inhibitors as eye drop solutions at pH values close to neutrality, in order to obtain more effective antiglaucoma drugs devoid of topical side effects, but the tail approach is a general one, which was also used for the design of antitumor sulfonamides among others.[9]

We recently developed new benzenesulfonamide CA inhibitors bearing thiourea tails, possessing interesting antiglaucoma properties via the topical route of administration.[10] Many of these derivatives showed very good water solubility, at nearly neutral pH values, whereas their hydrophobic properties (log *P*), and their accession rates across the cornea were demonstrated to be those appropriate to act as efficient topical intraocular pressure (IOP) lowering agents. Some of these new sulfonamides acted as effective enzyme inhibitors in vitro against the human isozymes hCA II and hCA I. Several of the most active inhibitors strongly lowered IOP pressure in normotensive and glaucomatous rabbits showing a prolonged duration of action as compared to the actual drug dorzolamide.[10] For these reasons these new compounds could be used as leads for the develop-

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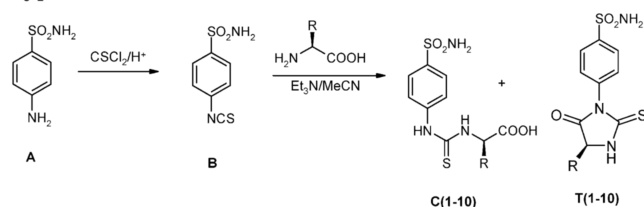
Scheme 1. Solution Approach to the Synthesis of 4-Sulfamoylphenylthioureas **C1−C10**, When Thiohydantoins **T1−T10** Are Also Formed as Byproducts



ment of focused libraries of new CA inhibitors in which different tails can modulate the physicochemical properties of the resulting sulfonamide, but an undesired aspect in this series of derivatives consisted in the formation of thiohydantoins as side products, in addition to the desired thiourea derivatives. This leads to low reaction yields of the desired CA inhibitors, because of the formation of byproducts, and as a consequence, long and expensive steps of purification of the obtained mixtures. Thus, we decided to find an alternative synthetic strategy for these interesting thioureas, taking advantage of a solid-phase approach for such sulfonamides. Furthermore, an on-bead screening was developed based on the on-resin supported compounds tested for their CA inhibitory activity against human CA I, CA II, and CA IX isozymes. Thus, this first solid-phase synthesis of CA inhibitors ever reported is also instrumental for a screening of the resin-coupled sulfonamides, which allows an appreciable enhancement of efficiency in detecting compounds with the desired biological profile.

Results

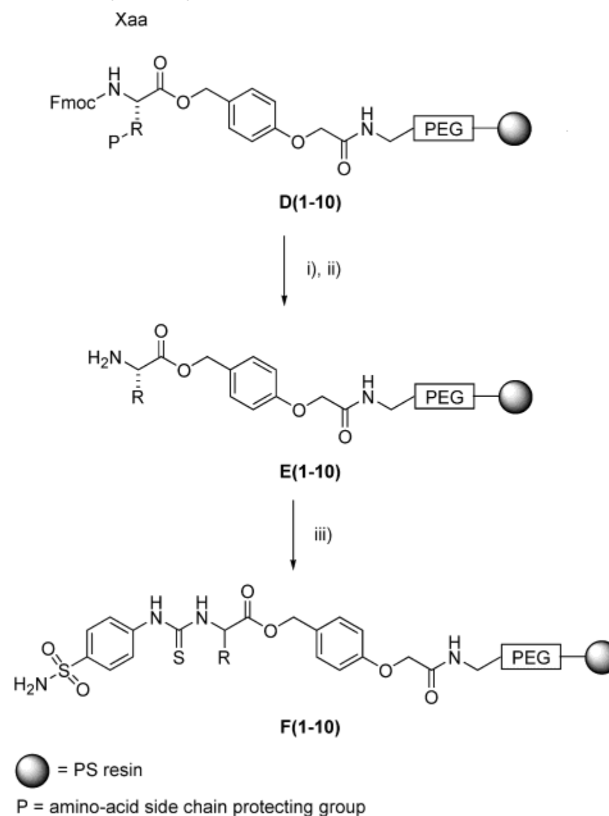
Chemistry. The solution and solid-phase synthetic strategies for obtaining the thiourea sulfonamide CA inhibitors are shown in Schemes 1 and 2.

The synthesis of the thiourea sulfonamide **C1−C10** is performed in solution heating in MeCN at reflux temperature a mixture of the amino acid and of 4-isothiocyanato-benzenesulfonamide **B**.[11] The reaction was completed in 2−6 h yielding a mixture of the desired thiourea **C1−C10** and of the corresponding thiohydantoins **T1−T10**.

The solid-phase synthesis was performed on a NovaSyn Tentagel resin functionalized with the amino acid orthogonally protected for the Fmoc/tBu solid-phase peptide synthesis. The synthesis was performed on the Fmoc-Xaa-TGA-resins **D1−D10**, with Xaa = Ala, Asn(Trt), Gln(Trt), Ile, Leu, Met, Phe, Pro, Trp(Boc), Val. Fmoc deprotection was performed by treating the resin with a solution of 20% piperidine in DMF. Side-chain deprotection of the H-Xaa-TGA-resins [Xaa = Asn(Trt), Gln(Trt), Trp(Boc)] was undertaken with 30% TFA in DCM. Thiourea formation was performed by treating the resin **E1−E10** with a solution of 4-isothiocyanatobenzenesulfonamide **B** in acetone/dioxane at 65 °C for 3 h. The reaction was monitored by the Kaiser test.[12] All the syntheses in the solid phase showed a negative Kaiser test after 3 h of heating, demonstrating the absence of free amino groups.

CA Inhibition. The obtained inhibitors **C1−C10** and the corresponding on-resin intermediates **F1−F10** which were also assayed for inhibition activity against isozymes hCA I and II are reported in Table 1. The typical inhibition curves are presented in Figure 1.

Scheme 2. Solid-Phase Approach to 4-Sulfamoylphenylthiourea Derivatives **F1−F10**[a]



[a] Reagents: (i) 20% piperidine in DMF (2 × 10 min); (ii) only for Xaa = Asn(Trt), Gln(Trt), Trp(Boc), 30% TFA in DCM (2 × 15 min); (iii) isothiocyanate **B** (1.2 equiv) in acetone/dioxane (1:1), 3 h, 65 °C. Amino acid Xaa = Ala, Asn, Gln, Ile, Leu, Met, Phe, Pro, Trp, Val **(1−10)**, numbering as in Scheme 1.

Table 1. CA Inhibition Data with Standard Inhibitors, 4-Isothiocyanatobenzenesulfonamide **B**, and the Thiourea Sulfonamide Inhibitors Obtained by Solution **C1−C10** and Solid Phase Synthesis **F1−F10**

solution synthesis			on-resin synthesis		
	K_i (nM)			K_i (nM)	
compound	hCA I[a]	hCA II[a]	compound	hCA I[a]	hCA II[a]
dorzolamide	50000	9			
acetazolamide	900	12			
methazolamide	780	14			
ethoxzolamide	25	8			
dichlorophenamide	1200	38			
B	5000	185			
C1	54	12	**F1**	58	16
C2	27	6	**F2**	29	4
C3	40	9	**F3**	53	11
C4	22	4	**F4**	25	5
C5	21	5	**F5**	23	7
C6	27	4	**F6**	31	5
C7	30	6	**F7**	42	8
C8	110	25	**F8**	197	46
C9	47	11	**F9**	64	20
C10	23	5	**F10**	31	5

[a] Human recombinant enzymes, by the esterase assay (4-nitrophenylacetate as substrate).[16] Errors were in the range of ±5−10% of the reported values, from three different assays.

Discussion

Chemistry. We have previously reported the synthesis of a large series of 4-sulfamoylphenylthioureas

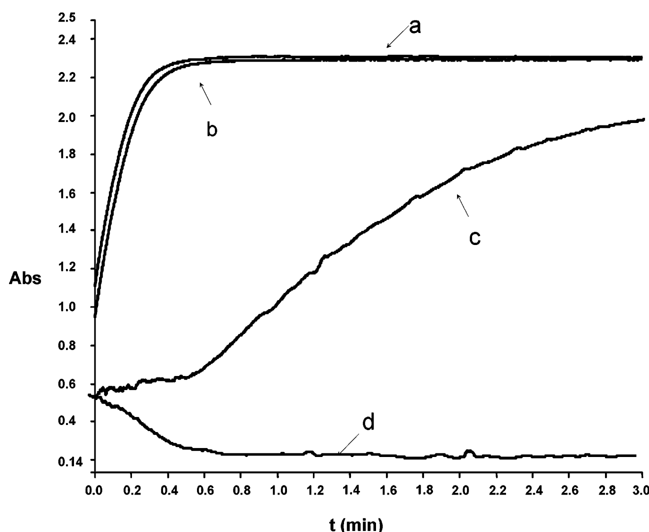


Figure 1. Example of typical absorbance vs time curves obtained following the hCA II esterase activity with the developed on-bead assay methodology. Trace a: hCA II 10^{-6} M. Trace b: hCA II 10^{-6} M + amino acid coupled resin **D**. Trace c: hCA II 10^{-6} M + 10^{-7} M inhibitor **F7**. Trace d: hCA II 10^{-6} M + 10^{-6} M inhibitor **F7**.

incorporating different amino acid/oligopeptide functionalities, comprising also the compounds **C1**−**C10** reinvestigated here.[10] The synthesis was performed in solution, by coupling the 4-sulfamoyl-benzeneisothiocyanate **B** (obtained from sulfanilamide **A** and thiophosgene)[11] with the unprotected amino acid in MeCN, at reflux temperature and in the presence of base (triethylamine) as a catalyst (Scheme 1). However, we observed that, during these syntheses, the formation of the desired thiourea **C1**−**C10** was accompanied by the formation of various amounts of thiohydantoins of the type **T1**−**T10** as side products. As a consequence, the solution-phase approach was limited by time-consuming steps for the isolation and purification of the desired products **C1**−**C10**, usually by means of HPLC dramatically affecting the final yield.[10]

In order to avoid thiohydanthoin formation, we decided to explore a solid-phase approach for the synthesis of 4-sulfamoylphenylthioureas derivatized with different amino acids. In this particular case, the solid-phase strategy was found to provide not only an efficient synthetic tool but also a powerful instrument for the development of a high-throughput on-bead screening of CA inhibitors.

Considering the synthetic pathway, thiourea formation is widely described and applied, both in solution and in the solid phase, for peptide and protein sequencing by Edman degradation, by reaction of phenyl isothiocyanate with the N-terminal amino group of a peptide generating the phenylthiocarbamoyl derivative.[13] In the Edman degradation, the thiourea-labeled N-terminal amino acid is subsequently transformed under acidic conditions via an intramolecular cyclization, into the thiohydantoin derivative of the N-terminal amino acid that is then separated from the peptide fragment. This technique performed in the solid phase allows one to obtain in acidic condition the thiohydantoin compound of the N-terminal amino acid in solution, and the peptide fragment still anchored to the resin.[14] Thus the peptide fragment anchored to the solid support can be easily recovered without compromising its purity only by filtering the thiohydantoin derivative and washing the resin.

Therefore, taking advantage of the solid-phase strategy, on-resin thiourea can be synthesized avoiding thiohydantoin formation. Once the thiourea is formed, this compound is the only product remaining anchored to the resin. In fact, the thiohydantoin derivative is obtained in solution, if an acid-catalyzed cleavage from the resin occurs. On the other hand, standard cleavage conditions from the resin (95% TFA) should lead to a mixture of the desired thiourea derivatives and of the corresponding thiohydantoin. Consequently, as the solid-phase strategy avoids time-consuming purification steps of the final products and provides thioureas as unique on-resin products, a solid-phase approach will be an effective tool for the synthesis of libraries of 4-sulfamoylphenylthioureas only if these compounds can be directly screened on resin.

An on-bead binding assay is a fruitful method for the screening of libraries as it allows efficient and rapid detection of lead compounds.[15] Particular attention should be focused on the biological tests performed on the on-resin libraries of 4-sulfamoylphenylthioureas in order to obtain screening results comparable to our standard CA inhibitory assays in solution.[10] Therefore, this work deals with the optimization of the parameters involved in the synthetic pathway and in the biological tests, for further setup and high-throughput on-resin screening of libraries of 4-sulfamoylphenylthioureas derivatized with single amino acids or with oligopeptide residues. As we previously reported that the most active CA inhibitors contained hydrophobic (Val, Leu, Ile), aromatic (His, Phe, Tyr), or dicarboxylic amino acids (Asp, Glu, and their corresponding amides Asn, Gln),[10] we focused our efforts on the solid-phase preparation of such 4-sulfamoylphenylthiourea derivatives in order to compare the efficiency of the biological screening performed in the solid phase with the solution one. Therefore, 4-sulfamoylphenylthioureas were derivatized with the following amino acids: Ala, Asn, Gln, Ile, Leu, Met, Phe, Pro, Trp, Val (Scheme 1). To further perform an on-resin biological assay a crucial step is the choice of the resin, which has to be compatible with the solid-phase synthetic strategy and with the aqueous conditions to be used for the screening process. The commercially available NovaSyn TGA resin (polyethylene oxide grafted onto a low cross-linked polystyrene gel-type matrix, Calbiochem-NovaBiochem, Switzerland), derivatized with the TFA-labile 4-hydroxymethylphenoxyacetic linker and functionalized with the appropriate Fmoc-amino acid, has been found to be an efficient solid support (Scheme 2).

Fmoc-Xaa-TGA resins **D1**−**D10** [Xaa = Ala, Asn(Trt), Gln(Trt), Ile, Leu, Met, Phe, Pro, Trp(Boc), Val], orthogonally protected for the Fmoc/tBu solid-phase peptide synthesis, were treated with a solution of 20% piperidine in DMF to deprotect the amino function from the Fmoc group (Scheme 2). For the H-Xaa-TGA resins, with Xaa = Asn(Trt), Gln(Trt), Trp(Boc), a subsequent deprotection of the amino acid side chain with a solution of 30% TFA in DCM was performed before the thiourea formation, in order to recover the functional groups present on the amino acid side chain with a putative role in the

expression of their biological activity. While the reaction in solution between the amino acids and 4-sulfamoylbenzeneisothiocyanate **B** for the formation of the thiourea (Scheme 1) required prolonged reflux in MeCN (2−6 h) in the presence of high amounts of triethylamine,[10] we found that the solid-phase approach led to completion of the reaction in acetone/dioxane at 65 °C in 3 h (Scheme 2). The solid-phase reactions were followed by the Kaiser test until complete disappearance of free amino groups on the resins, and the 4-sulfamoylphenylthiourea derivatives **F1**−**F10** were identified on-resin by FT-IR (data were in accordance with the compounds previously characterized in solution).[10]

CA Inhibition Studies. The thioureas **C1**−**C10** and the resin-bound sulfonamides **F1**−**F10** were tested for their CA inhibitory activity against isozymes hCA I and hCA II by a spectrophotometric assay evaluating the esterase activity of the selected enzymes.[16] The on-resin assay of the focused library avoided the cleavage of the thioureas from the resin, therefore circumventing thiohydantoin formation and accelerating the process of a powerful inhibitor selection. This is the first reported on-bead assay in a library of CA inhibitors.

The enzyme activity screening strategy consisted in evaluating the esterase activity of the CAs through spectrophotometric analysis. In detail the kinetic of hydrolysis of the ester *p*-nitrophenylacetate by the selected enzyme was recorded, following the production during time of the species *p*-nitrophenolate, which absorbs at 400 nm. The TGA resin was chosen as a solid support for our synthetic strategy, for its ability to swell in aqueous medium due to the presence of polyethyleneglycol (PEG) moieties in its structure. This feature allows the appropriate contact between the enzyme molecules and the free sulfonamidic groups of the resin-bound inhibitors in solution. Therefore this resin does not prevent the interaction between the inhibitors and the CAs, and it favors the recording of the kinetics of the enzyme.

In Figure 1, the absorbance at 400 nm vs time curves for the kinetic analysis of hCA II alone (trace a), in the presence of the amino acid coupled to resin **D** (trace b), and in the presence of 10^{-6} and 10^{-7} M on-resin inhibitor **F7** (traces c and d, respectively) are reported as examples of the obtained results. This plot shows that the kinetics of the target isozyme is not affected by the polymeric support used for the solid-phase synthesis (traces a and b are almost identical). Therefore, the K_i values calculated for each on-resin compound depend only on the inhibitory power induced by the presence of the sulfonamide derivative on the resin, and this power varies dose-dependently with the concentration of the inhibitor used.

In Table 1 the K_i values for compounds **C1**−**C10** and **F1**−**F10** are reported in comparison with those determined for the isothiocyanate sulfonamide **B** and for some standard, clinically used sulfonamide CA inhibitors. Clearly, **F1**−**F10** are esters, whereas **C1**−**C10** are acids, but since these groups are presumed not to directly interact with the zinc ion of the enzyme, we consider that the comparison of these types of compounds is appropriate for the goal of our work, i.e., to develop a rapid on-resin screen for detecting potent CA inhibitors. From data of Table 1 it may be seen that there is a very close parallelism between the inhibition data of the resin-coupled sulfonamides (**F1**−**F10**) and the corresponding free sulfonamides, obtained via the solution synthesis, of types **C1**−**C10**, against both tested CA isozymes, hCA I and hCA II. Thus, differences of activity are generally within the range of experimental errors (±5−10% of the reported values) or the slightly weaker inhibitory power of the **F**-type compounds may be due to their binding to the resin (for example compare **C8** with **F8** or **C9** with **F9**, which represent the most divergent values). Nevertheless, these differences reflect indeed the inhibitory power of the corresponding CA inhibitors, since it may be clearly seen that, in both series of derivatives (the **C**-type and **F**-type), the nature of the amino acid residue influences practically in the same manner the inhibitory power against both hCA I and hCA II. This is indeed a very important result, as it proves that the on-bead testing of CA inhibitory efficiency in a library of thioureido-benzene-sulfonamides may be done successfully, without the need of deprotecting all the obtained derivatives (and thus an important gain of efficiency, a reducing of time and costs for detecting good hits, etc). It is thus possible to detect immediately the best inhibitors even in very large libraries of such compounds, and at the end selecting for cleavage and purification only those derivatives displaying an optimal biological activity against the target isozyme. Indeed, this approach exemplified here for the blood cell isozymes hCA I and II (the most widely spread in humans)[1−3] may be adapted to other isozymes, such as the mitochondrial one, hCA V, for which very recently the first inhibitors were reported (which may lead to compounds effective in the prevention or treatment of obesity),[22] or the tumor-associated one (hCA IX), for which we recently reported effective inhibitors, which may lead to novel therapeutic approaches for tumors.[23]

The data of Table 1 also show that these compounds inhibit better hCA II than hCA I, both when they are in solution and when they are attached to the resin. Thus, inhibition constants were in the range 25−197 nM against the hCA I (for the **F**-type compounds) and 21−110 nM against the same isozyme, for the **C**-type compounds. For hCA II, the inhibition constants were in the range 4−46 nM for the **F**-type compounds, and 4−25 nM for the **C**-type compounds. In both series, the best hCA I inhibitors were the derivatives of Ile, Val, and Leu, whereas the best hCA II inhibitors were the derivatives of Asn, Ile, Leu, Met, Phe, and Val.

Conclusions. We report here the solid-phase synthesis of a focused library of 4-sulfamoylphenylthioureas and their on-resin screening as CA inhibitors with pharmacological applications. The solid-phase strategy allowed the synthesis of the selected sulfonamides avoiding the side reactions and the drawbacks typical of a solution approach (thiohydantoin formation and long purification steps). Moreover, using the solid-phase protocol, we set up a new on-resin assay methodology that allowed rapid selection of potent CA inhibitors and discrimination of their inhibitory activity in the range of low nanomolar concentrations. These results may enhance the synthesis and design of libraries of sulfonamides that fit a set of prefixed parameters to be used as possible drugs.

Experimental Section

General. Sulfanilamide, thiophosgene, and amino acids used in the synthesis were highest purity, commercially available compounds (from Sigma-Aldrich, Fluka, Merck, or Acros). Acetonitrile, acetone (Merck), or other solvents used in the synthesis were doubly distilled and kept on molecular sieves in order to maintain them in anhydrous conditions. Peptide grade DMF was obtained from Scharlau (Barcelona, Spain). All reactions were monitored by thin-layer chromatography (TLC) using 0.25-mm precoated silica gel plates (Merck).

Commercially available NovaSyn Fmoc-Xaa-TGA-resin, functionalized with the amino acids protected for the Fmoc/tBu solid-phase peptide synthesis, Xaa = Ala, Asn(Trt), Gln(Trt), Ile, Leu, Met, Phe, Pro, Trp(Boc), Val, were purchased from Calbiochem-Novabiochem AG (Laufelfingen, Switzerland). The NovaSyn TGA resin (polyethylene oxide grafted onto a low cross-linked polystyrene gel-type matrix) is derivatized with the TFA-labile 4-hydroxymethylphenoxyacetic linker.

^{1}H NMR spectra were recorded in DMSO-d_6 as solvent, with a Bruker CPX200 or Varian 300 instrument. Chemical shifts are reported as δ values, relative to Me_4Si as internal standard. FT-IR spectra on KBr pellets were recorded on a Perkin-Elmer model 881 spectrometer.

Solid-phase reactions were performed in the manual synthesizer PLS 4x4 (Advanced ChemTech), in 20 mL Teflon reactors, equipped with a filter. Mixing is provided by vortex, while filtration is performed by connecting the reactor to a vacuum pump. This instrument allows solid-phase syntheses at different temperatures.

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.[17] Cell growth conditions were those described in the literature,[18] and enzymes were purified by affinity chromatography according to the method of Khalifah et al.[19] 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. [20,21]

General Procedure for Preparation of 4-Sulfamoylphenylthioureas in Solution C1−C10. The synthesis of 4-isothiocyanatobenzenesulfonamide **A** has already been reported in 1946 by McKee and Bost,[11] in the search of more effective antibacterial sulfonamides. Recently, our group has used this highly versatile compound for the preparation of sulfonamides possessing CA inhibitory properties.[10] Compound **B** has been obtained by the reported literature procedure,[11] from sulfanilamide **A** and thiophosgene, and was subsequently reacted with amino acids **1**−**10** leading to the known thioureas **C1**−**C10** respectively (Scheme 1).[10,11] In detail an amount of 0.53 g (2.5 mmol) of 4-isothiocyanato-benzenesulfonamide **B** and the stoichiometric amount of amino acid were suspended in 50−100 mL of dry acetone or acetonitrile and heated at reflux for 2−8 h (TLC control). The solvent was evaporated, and the crude product either recrystallized from ethanol or ethanol−water, or purified by preparative HPLC in the case when the reaction mixture contained appreciable amounts of impurities (as evidenced by TLC). Conditions used for the purification were as follows: C_{18} reversed-phase μ-Bondapak or Dynamax-60A (25 × 250 mm) preparative columns; 90% acetonitrile/8% methanol/2% water, 30 mL/min.

General Procedure for the Solid-Phase Synthesis of 4-Sulfamoylphenylthioureas F1−F10. A series of thiourea derivatives **F1**−**F10** were synthesized in the solid phase starting from the functionalized NovaSyn Fmoc-Xaa-TGA-resin **D1**−**D10**, Xaa = Ala, Asn(Trt), Gln(Trt), Ile, Leu, Met, Phe, Pro, Trp(Boc), Val. The resin (0.20 mmol/g) placed in the PLS 4x4 reactor was swollen for 40 min in DMF (1 mL × 100 mg of resin). After filtration, Fmoc deprotection was performed with a solution of 20% piperidine in DMF (2 × 15 min). The resin was washed with DMF (3 × 2 min) and DCM (2 × 2 min). For H-Xaa-TGA-resin with Xaa = Asn(Trt), Gln(Trt), Trp(Boc), the side-chain deprotection was performed by treating the resin with a solution of 30% TFA in DCM (2 × 15 min).

A solution of the isothiocyanate **B** (1.2 equiv) in acetone/dioxane (1:1) was added to the resin **E1**−**E10**, which was vortexed at 65 °C for 3 h. The reaction was monitored by a ninhydrin Kaiser test.

The resin was washed with DCM (3 × 2 min) and acetone (2 × 2 min), and then dried under vacuum.

***N*-[Fluorenylmethoxycarbonyl]-l-methionine NovaSyn TGA-resin bound (D6):** FT-IR (KBr) cm^{-1} 3024, 1717, 1651, 1350, 1250.

l-Proline NovaSyn TGA-resin bound (E8): FT-IR (KBr) cm^{-1} 3025, 1636, 1351, 1250.

***N*-[[[4-(Aminosulfonyl)phenyl]amino]thioxomethyl]-L-alanine NovaSyn TGA-resin bound (F1):** FT-IR (KBr) cm^{-1} 3024, 1751, 1648, 1352, 1251.

***N*-[[[4-(Aminosulfonyl)phenyl]amino]thioxomethyl]-L-asparagine NovaSyn TGA-resin bound (F2):** FT-IR (KBr) cm^{-1} 3025, 1746, 1723, 1684, 1351, 1252.

***N*-[[[4-(Aminosulfonyl)phenyl]amino]thioxomethyl]-L-glutamine NovaSyn TGA-resin bound (F3):** FT-IR (KBr) cm^{-1} 3025, 1742, 1718, 1684, 1343, 1242.

***N*-[[[4-(Aminosulfonyl)phenyl]amino]thioxomethyl]-L-isoleucine NovaSyn TGA-resin bound (F4):** FT-IR (KBr) cm^{-1} 3024, 1750, 1653, 1343, 1242.

***N*-[[[4-(Aminosulfonyl)phenyl]amino]thioxomethyl]-L-leucine NovaSyn TGA-resin bound (F5):** FT-IR (KBr) cm^{-1} 3025, 1756, 1675, 1353, 1253:

***N*-[[[4-(Aminosulfonyl)phenyl]amino]thioxomethyl]-L-methionine NovaSyn TGA-resin bound (F6):** FT-IR (KBr) cm^{-1} 3024, 1750, 1651, 1350, 1250.

***N*-[[[4-(Aminosulfonyl)phenyl]amino]thioxomethyl]-L-phenylalanine NovaSyn TGA-resin bound (F7):** FT-IR (KBr) cm^{-1} 3025, 1751, 1685, 1344, 1248.

***N*-[[[4-(Aminosulfonyl)phenyl]amino]thioxomethyl]-L-proline NovaSyn TGA-resin bound (F8):** FT-IR (KBr) cm^{-1} 3025, 1756, 1652, 1348, 1249.

***N*-[[[4-(Aminosulfonyl)phenyl]amino]thioxomethyl]-L-tryptophane NovaSyn TGA-resin bound (F9):** FT-IR (KBr) cm^{-1} 3025, 1735, 1675, 1351, 1251.

***N*-[[[4-(Aminosulfonyl)phenyl]amino]thioxomethyl]-L-valine NovaSyn TGA-resin bound (F10):** FT-IR (KBr) cm^{-1} 3023, 1755, 1651, 1352, 1250.

CA Inhibition Screening. The initial rates of 4-nitrophenylacetate hydrolysis catalyzed by different CA isozymes alone or in the presence of the inhibitor are monitored spectrophotometrically, at 400 nm, in a quartz cuvette through a Perkin-Elmer Lambda 20 Bio spectrophotometer.[16] Stock solutions of inhibitor (10^{-2} M) were prepared using DMSO as solvent, and then dilutions of each inhibitor from 1 mM up to 1 nM were done thereafter with the appropriate buffer (HEPES 0.01 M, TRIS hydrochloride 0.01 M, Na_2SO_4 0.1 M, pH 7.50). Then 1 mL assay samples were prepared using 100 μL of inhibitor at a certain concentration, 890 μL of buffer, and 10 μL of enzyme. Final enzyme concentrations in the assay samples were 1 μM for hCA II and 10 μM for hCA I. Inhibitor and enzyme solutions were preincubated together for 15 min at room temperature prior to assay, in order to allow for the formation of the E−I complex. Stock solution of substrate was prepared in anhydrous acetonitrile; the substrate concentration was 2 × 10^{-2} M. A molar absorption coefficient ϵ of 18400 M^{-1} cm^{-1} was used for the 4-nitrophenolate formed by hydrolysis, in the conditions of the experiments (pH 7.50), as reported in the literature.[16] Nonenzymatic 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. The inhibition constant K_I was determined as described by Pocker and Stone.[16] Analysis of absorbance spectra was performed using UV-Winlab software (Perkin-Elmer). Both soluble and on-resin sulfonamides reported here have been tested as described above.

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