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Inhibition studies of new ureido-substituted sulfonamides incorporating a GABA moiety against human carbonic anhydrase isoforms I–XIV

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ABSTRACT

Reaction of γ-Boc-GABA, prepared by protecting the γ-amino moiety of the amino butyric acid with the *tert*-butyloxycarbonyl (Boc) protecting group, with 4-methyl/ethyl benzenesulfonamide, followed by removal of the Boc protecting group in 3 M HCl afforded the corresponding hydrochlorides, which were further derivatized by reaction with a varying of aryl isocyanates to give a new classes of ureido substituted benzenesulfonamide containing a GABA moiety. Inhibition studies of the human carbonic anhydrase (CA, EC 4.2.1.1) isoforms, CA I–XIV with these new compounds revealed that they possess moderate–weak inhibition potency against hCA III, IV, VA, VI and XIII, rather efficient inhibitory power against hCA I, VI, and IX, and excellent inhibition of the physiologically relevant hCA II and VII, as well as of the two tumor-associated isoforms CA IX and XII. The inhibition profile of the new ureido-substituted benzenesulfonamides reported here is thus very different from the corresponding ureido-substituted analogs incorporating sulfanilamide, which were previously investigated as inhibitors of some of these enzymes.

1. Introduction

The discovery of isoform-selective CA inhibitors or at least organ-specific targeting inhibitors would represent one of the most important aims to be achieved by the research on CAs which only recently became very intense. In order to obtain new drugs with less undesired side effects mostly produced by the first generation of inhibitors and for physiological studies in which specific/selective inhibitors may constitute valuable tools for understanding the physiology/physiopathology of hCA isoforms,[1,2] new class of inhibitors containing already known effective moiety as CAs in their structure have been designed and synthesised.

These novel types of drugs would mainly have the feature of being isoenzyme selective inhibitors. However, due to the similarity among the related isoforms of the same class of enzyme, the selectivity of CAs is essential and difficult to achieve during the design of either potential drugs or pharmacological tools. Indeed, the family of carbonic anhydrases (CAs, EC 4.2.1.1), which is involved in a variety of physiological and pathological processes of remarkable interest, is ubiquitous metallo-enzymes mainly catalysing the reversible hydration of CO_2 to HCO_3^- and H^+[3].

This class of enzyme is present either in prokaryotes or eukaryotes and are encoded by six distinct evolutionarily non-related gene families: alpha, beta, gamma, delta, zeta and eta.[4] All human CAs (hCAs) belong to the alpha-class; up to now, fifteen isozymes have been recognized, among these only twelve are catalytically active (CAs I–IV, CAs VA–VB, CAs VI–VII, CA IX and CAs XII–XIV), whereas the CA-related proteins (CARPs) VIII, X and XI resulted without any catalytic activity. The different CA isoforms widely vary in their inhibition, kinetic properties, pattern of expression in various tissues and cellular localization. Indeed CA I, II, III, VII and XIII are expressed in the cytosol, CA IV, IX, XII and XIV are associated with the cell membrane, CA VA and VB reside in mitochondria, whereas CA VI is secreted in saliva and milk.

Due to the important roles of carbonic anhydrases in a variety of physiological processes and the association of abnormal levels and/or activities of these enzymes to different human diseases (such as glaucoma, osteoporosis, neurological disorders, cancer, etc.), the CA isozymes have become in the last years an interesting target for the design of inhibitors or activators with biomedical applications. Originally CA inhibitors (CAIs) were clinically used mainly as diuretics, anti-glaucoma and anti-epileptics, while the novel

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generation compounds are undergoing clinical investigation as anti-obesity or anti-tumor drugs/diagnostic tools.[3–10] The sulfonamides constitute the major, the most clinically used and most studied family of CAIs.[3,10–13] However, none of the currently CA inhibitors in clinical use shows selectivity for a specific isozyme. Thus, the development of isozyme-specific CA inhibitors would be extremely helpful to discover novel classes of drugs lacking of a range of undesired side-effects.

Only recently a large series of 4-RNHCONH-substituted benzenesulfonamide derivatives was synthesised and investigated as inhibitors of the cytosolic isoform hCA I and II (h = human) and the two transmembrane tumour-associated isoforms IX and XII by our group.[14]

It has been reported that the inhibition potency of similar sulfonamides containing an ureido group changes by varying the orientation of their R-ureido moieties when the inhibitor was bound within the enzyme active site. Indeed, X-ray crystallography studies have shown a highly variable orientation of the R-ureido moieties when the inhibitor was bound within the enzyme active site.[14] Due to the specific chemical nature of the varying R-ureido linker, the compounds can adopt different orientations in the X-ray crystal structures. Therefore, a strong correlation has been observed between the binding pattern, the R group orientation and the CA II inhibitory properties for this small series of compounds, where R was 4-fluorophenyl, pentafluorophenyl, 2-isopropylphenyl, 3-nitrophenyl, and cyclopentyl.[14]

On the other hand, it has been also reported new sulfonamide derivatives with high selective inhibition potency against some CA isoforms over the most common and ubiquitous cytosolic ones.[15] Indeed, new sulfonamide derivatives containing amino acid or GABA moiety resulted to be effective and selective inhibitors of the tumor-associated carbonic anhydrase XII.[16]

Therefore, it appeared of great interest to further explore this combination of scaffolds for obtaining new class of CAIs. Here we extend our earlier investigations on 4-ureido-substituted sulfonamide and GABA containing CAIs and report the synthesis and inhibition studies against all human (h) isoforms hCA I–XIV, of a small series of new six similar ureido-substituted sulfonamide CAIs containing this time an elongation of their main skeleton such as a GABA moiety.

2. Results and discussion

2.1. Chemistry

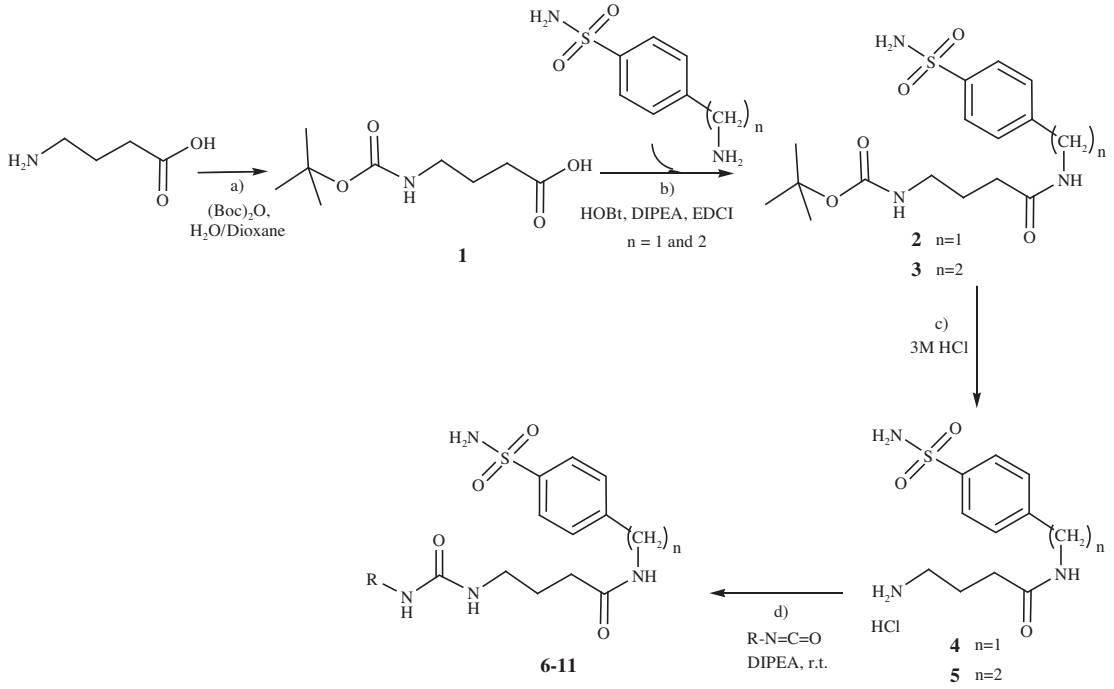
Starting from GABA, by protecting the γ-amino moiety with the *tert*-butyloxycarbonyl (Boc) protecting group through a procedure already reported in literature[16] to give the Boc-GABA **1**, which was consequently coupled with 4-methyl/ethyl benzenesulfonamide, the γ-Boc-GABA-substituted sulfonamides **2** and **3** were prepared (Scheme 1). After removal of the Boc protecting group in 3 M HCl, the hydrochlorides **4** and **5** were treated with aryl isocyanate A1–4 (R—N=C=O) (Scheme 1).

It has been showed that the benzenesulfonamides incorporating 4-substituted ureido moieties act as potent hCA II inhibitors with potencies that correlate well with the orientation of the R moiety present in the ureido tail of the compound. Since it has also been reported earlier that other classes of ureido-substituted benzenesulfonamides showed to be isoform-selective compounds,[14] we considered interesting to continue and expand this investigation by synthesising new ureido-benzene sulfonamides derivatives containing this time a GABA as main skeleton.

Therefore, we report here the synthesis of a new series of ureido-substituted compounds **6**–**11** prepared by reaction of **4** or **5** with aryl isocyanates A1–A4 (Scheme 1). The chemical diversity was generated by varying the nature of the starting isocyanate, A1–A4 (Table 1).

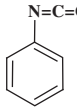
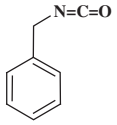
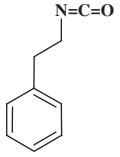
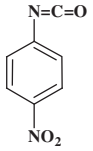
The difference of CA isoforms is mainly represent by the changing of their amino acid sequences in the external portion of their active sites; therefore, compounds with an R group moiety that can strongly interact with this region will show a better selectivity profile for the various isoforms.[13]

This hypothesis was confirmed by the work done earlier in our group,[12] indeed it has been reported that some ureido-substituted



Scheme 1. Preparation of sulfonamides **6**–**11** by reaction of 4-amino-*N*-(4-sulfamoyl-benzyl)-butyramide hydrochloride (*n* = 1) (**4**) or 4-amino-*N*-[2-(4-sulfamoyl-phenyl)-ethyl]-butyramide hydrochloride (*n* = 2) (**5**) with isocyanates (R-NCO) in presence of diisopropylethylamine (DIPEA), in acetonitrile (dry).

Table 1
Isocyanates used for the preparation of compounds **6–11**

Isocyanates	R
A1	
A2	
A3	
A4	

sulfonamides show selective inhibition of isoform hCA I over the dominant one hCA II. Indeed, it has been shown that the benzenesulfonamide moiety of these 4-ureido substituted derivatives binds in the usual manner, coordinating to the zinc ion as SO_2NH- group. Indeed, the phenylsulfamoyl moiety of the inhibitor was highly imposable between all these adducts occupying the middle of the active site cavity and binding in the usual way.[3,14]

However, the different R moieties present in this series of compounds were showed to occupy various subpockets of the enzyme active site when bound to the external part of the enzyme, and none of them was imposable with each other. Due to the varying flexible nature of the R ureido linker connecting the benzenesulfonamide of these compounds, a selective inhibition of hCA I isoform over the dominant one hCA II was thus demonstrated. Normally, it is hCA II having higher affinity for sulfonamides compared to hCA I, at least for most of the clinically used derivatives.[1–3]

Our intent is thus to discover a new lead compounds which will show a selectivity against hCA isoforms. The new compounds were characterized by ^1H and ^{13}C NMR as well as by mass spectroscopy, confirming their structures (see Section 4 for details).

2.2. CA inhibition

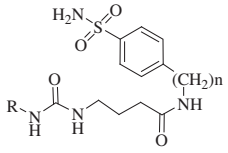
The inhibition studies of all mammalian CA isoforms with the new ureido-substituted benzenesulfonamides derivatives **6–11**, and the clinically used, standard CAIs such as acetazolamide (**AAZ**)[17] have been hereby reported. The carbonic anhydrase isoforms included in the study were cytosolic (hCA I, II, III, VII and XIII), membrane-bound (CA IV) or transmembrane ones (hCA IX, XII and XIV), mitochondrial (CA VA) and secreted (CA VI) and all of them are involved in a host of important physiological and pathological functions in vertebrates.[3–10]

The following structure activity relationship (SAR) has been obtained from data of Table 2, showing the inhibition of the CA isoforms with the new group of ureido-substituted benzenesulfonamide **6–11**:

(i) The slow cytosolic isoform hCA I was moderately inhibited by compounds **6**, **8** and **9**, with inhibition constants in the range of 214–373 nM (comparable to those of the clinically used drug acetazolamide, **AAZ**, 5-acetamido-1,3,4-thiadiazole-2-sulfonamide). The best hCA I inhibitors were the phenyl and 4-nitro substituted phenyl ureido derivatives of 4-aminoethylbenzenesulfonamide (**7** and **11**), with K_Is of 32–41.8 nM, as well as the ethylbenzene derivative of 4-aminomethylbenzenesulfonamide (**10**) (K_I of 52.6 nM). Although the compounds reported here contain a similar substitution pattern at the R moiety, it is interesting to notice that the phenyl 4-aminoethylbenzenesulfonamide derivate **7** was about 10-fold more effective as an hCA I inhibitor compared to its shorter congener **6**.

(ii) Against the physiologically dominant isoform hCA II; derivatives **6–11** behaved as very strong inhibitors with K_Is in the range of 4.9–41 nM. The slightly less effective compound was the benzyl ureido derivative of the ethylbenzensulfonamide **9** with a K_I value of 41 nM, whereas the remaining ones were all quite similar and highly effective hCA II inhibitors, with inhibition constants in a very narrow

Table 2
Inhibition data of human CA isoforms hCA I–XIV with sulfonamides **6–11** reported here and the standard sulfonamide inhibitor acetazolamide (**AAZ**) by a stopped flow CO_2 hydrase assay[17]



6-11

N°	*n*	R	K_I (nM)*										
			hCA I	hCA II	hCA III	hCA IV	hCA VA	hCA VI	hCA VII	hCA IX	hCA XII	hCA XIII	hCA XIV
6	1	C_6H_5	373.4	6.0	>21000	233.19	156.1	>120	50.6	46	37.4	>240	238.6
7	2	C_6H_5	32.2	4.9	>21000	438.95	346.7	>120	24.0	41.6	6.2	>240	65.8
8	1	$C_6H_5CH_2$	359	6.1	>21000	879.80	329.8	>120	253.5	456.6	58.3	>240	344.0
9	2	$C_6H_5CH_2$	214.4	41	>21000	779.70	359.4	>120	28.7	106.4	50.7	>240	70.9
10	1	$C_6H_5(CH_2)_2$	52.6	6.9	>21000	397.89	141.7	>120	21.9	300.9	43.5	>240	48.2
11	2	$4\text{-}NO_2C_6H_4$	41.8	6.8	>21000	737.69	123.8	>120	17.7	138.7	43	>240	59.0
AAZ[a]	–	–	250	12	20000	74	63	11	2.5	25	5.7	18	41

[a] From Ref. 1.
* Mean from 3 different assays, by a stopped flow technique (errors were in the range of ±5–10% of the reported values).

range (4.9–6.9 nM). Therefore, SAR is almost impossible to define as all substitution patterns lead to highly effective inhibitors of this isoform (Table 2).

(iii) Isoforms hCA III and IV were the ones which were less inhibited by the compounds investigated here. Most probably, due to the presence of a bulky side chain such as Phe198 in the centre of the active site of this isoform, it is well known[18] that hCA III is relatively insensitive to inhibition by bulky sulfonamides. Indeed, the residue of phenylalanine inside the active side of the enzyme may lead to steric clashes with the scaffold of the molecules. This hypothesis is also confirmed by the data reported in Table 2, as all the ureido substituted benzenesulfonamides were inefficient inhibitors (K_I >21 μM). However, it is also interesting to notice how acetazolamide is also a very weak hCA III inhibitor (Table 2) with K_I in the same micromolar range.

The membrane-bound isoform hCA IV was also poorly inhibited by all compounds **6**–**11** reported here, with K_Is in the range 233.2–879.8 nM which were anyhow about 10 times much weaker compared to **AAZ** (K_I of 74 nM)

(iv) The mitochondrial isoform hCA VA was moderate inhibited by half of the compounds (**6**, **10** and **11**) with K_Is values in the range 123.8–156.1 nM and thus about 2 fold higher than the standard used drug AAZ, whereas the derivatives **7**–**9** were ineffective inhibitors with K_Is in the range of 329.8–359.4 nM.

(v) The secreted isoform (in saliva, milk and tears) hCA VI was not inhibited significantly by all the sulfonamides reported here with K_Is over 120 mM. Therefore, the SAR is not possible to be observed as all the series of derivates have shown the inhibition constants in the same range (Table 2).

(vi) Generally, the sulfonamides investigated here behaved as moderate potency inhibitors (K_Is in the range of 17.7–50.6 nM) against the cytosolic isoform hCA VII. The best hCA VII inhibitor was the nitro derivative **11** with K_I of 17.7 nM which was anyhow much weaker compared to **AAZ** (K_Is of 2.5 nM).However, the least effective inhibitor was the benzyl ureido derivative of the 4-methylbenzenesulfonamide **8** (K_I of 253.5 nM) whereas for the remaining compounds **6**, **7**, **9** and **10** the SAR is again rather flat as most of them have the inhibition constants in a narrow range (21.9–50.6 nM). Considering the important difference of activity between the congeners **8** and **9** (which differ only by an extra CH_2 group in **9**) as hCA VII inhibitors—by a factor of about 10—it is obvious that very small structural differences in the scaffold of the inhibitor lead to major differences of activity.

(vii) Although the most efficient two hCA IX inhibitors were the only phenyl ureido benzenesulfonamide derivatives **6** and **7**, they showed to possess inhibition activity at nanomolar range in the same order of magnitude of AAZ. The remaining derivatives **8**–**11** incorporating bulkier substituents at ureido moiety were less effective hCA IX inhibitors, with inhibition constants in the range of 106.4–456.6 nM. However, It is also interesting to notice that generally the ureido-derivatives containing 4-aminoethylbenzenesulfonamide are higher effective hCAIX inhibitors than the shorter 4-aminomethylbenzenesulfonamide congeners (Table 2).

However, the second transmembrane isoform associated with tumors, hCA XII, was much better inhibited by the new ureido-substituted benzenesulfonamides investigated here, compared to hCA IX. All derivatives showed K_Is in a narrow range between 37.4–58.3 nM against this isoform (except for derivative **7** which acts as a potent inhibitor against hCA XII). Indeed, this tumor associated isoform was strongly inhibited by the phenyl ureido-derivative of the 4-aminoethylbenzenesulfonamide **7** with K_I of 6.2 nM, although its activity is in the same range as the clinically used inhibitor **AAZ.**

(viii) hCA XIII, a cytosolic isoform like hCA I, II, III and VII; was poorly inhibited by the sulfonamides investigated here. All the compounds have shown K_Is >240 nM, they were about ten times less active than the standard clinically used drug acetazolamide (**AAZ**) (Table 2).

(ix) The last transmembrane isoform, hCA XIV was moderately inhibited by compounds **6** and **8** (both of them incorporating methylbenzenesulfonamide moiety), with K_Is of 238.6–344 nM, and better inhibited by the remaining 4 derivatives (K_Is of 48.2–65.9 nM). The best hCA XIV inhibitor was the ethylbenzene ureido derivative of 4-aminomethylbenzenesulfonamide **10** (K_I of 48.2 nM), which has anyhow a similar inhibition activity of acetazolamide.

3. Conclusions

A small series of ureido substituted benzenesulfonamides containing a GABA moiety was synthesized, characterized and investigated for the inhibition of the 13 hCA earlier isolated and cloned isoforms from different groups, in particular the physiologically relevant isoforms hCA I and II, and the other cytosolic isoforms hCA III and XIII, as well as the two tumour-associated isoforms hCA IX and XII, with some other hCA isoforms involved in various pathologies, such as obesity (CA VA), epilepsy (CA VII and XIV).

Therefore, the inhibition studies of all the human carbonic anhydrase (CA, EC 4.2.1.1) isoforms, CA I–XIV with these new compounds revealed that they possess moderate–weak inhibition potency against hCA III, IV, VA, VI and XIII, rather efficient inhibitory power against hCA I, VI, and IX, and excellent inhibition of the physiologically relevant hCA I, II and VII, as well as of the two tumor-associated isoforms CA IX and XII. The inhibition profile of the new ureido-substituted benzenesulfonamides reported here is thus very different from the corresponding ureido-substituted analogs incorporating sulfanilamide, which were previously investigated as inhibitors of some of these enzymes. Some of these new compounds may thus represent interesting candidates for the development of novel antiglaucoma, edema, altitude sickness or antiepileptic drugs. Since the two transmembrane isoforms hCA IX and hCA XII were also strongly inhibited by the compounds reported here with K_I in low nanomolar range, this new class of ureido-substituted sulfonamide hCAIs may be a novel and interesting lead compounds for the development of more potent hypoxia induced cancer drug therapy.

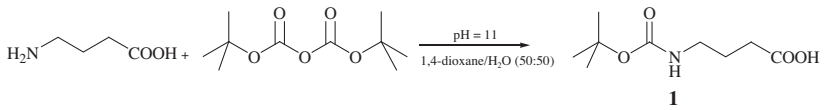
4. Experimental protocols

4.1. Chemistry

Anhydrous solvents and all reagents were purchased from Sigma–Aldrich, Alfa Aesar and TCI. All reactions involving air- or moisture-sensitive compounds were performed under a nitrogen atmosphere using dried glassware and syringes techniques to transfer solutions. Nuclear magnetic resonance (^{1}H NMR, ^{13}C NMR, DEPT-135, DEPT-90, and HSQC) spectra were recorded using a Bruker Avance III 400 MHz spectrometer in DMSO-d_6. Chemical shifts are reported in parts per million (ppm) and the coupling constants (J) are expressed in Hertz (Hz). Splitting patterns are designated as follows: s, singlet; d, doublet; sept, septet; t, triplet; q,

quadruplet; m, multiplet; br s, broad singlet; br m, broad multiplet; dd, double of double; td, triplet of double; tt, triplet of triplet; appd, apparent double; appt, apparent triplet. The assignment of exchangeable protons (N*H*) was confirmed by the addition of D_2O. Electron ionization mass spectra (70 eV) were recorded on a Hewlett–Packard 5989 Mass Engine Spectrometer. Analytical thin-layer chromatography (TLC) was carried out on Merck silica gel F-254 plates.

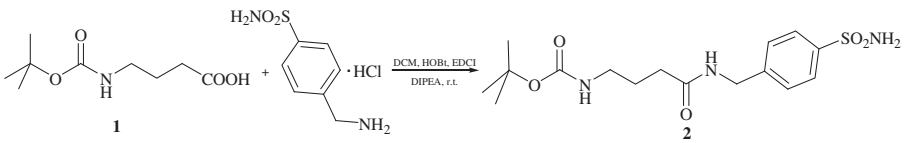
4.1.1. Synthesis of 4-*tert*-butoxycarbonylamino-butyric acid 1



A solution γ-amino butyric acid (1 g, 1.0 equiv) in a 1,4-dioxane/water (50:50) mixture (75 ml) was adjusted to pH 11 with 1.0 M NaOH aqueous solution and cooled down to −10 °C in a salt ice bath. Di-*tert*-butyl dicarbonate (2.1 g, 1.0 equiv) was then added and the pH maintained at 11 if necessary. The reaction mixture was then stirred overnight at room temperature. At the end of the reaction time the solution was acidified to pH 1 using a 3.0 M HCl solution. The solution was then extracted with ethyl acetate. The combined organic layers were dried over anhydrous sodium sulphate, filtered, and the solvent was then removed under reduced pressure to give 1.44 g of the pure product as colourless oil.

4-tert-Butoxycarbonylamino-butyric acid **1**: 73% yield; δ_H (400 MHz, DMSO-d_6): 6.84 (br t, 1H, N*H*, exchange with D_2O), 2.95 (td, 2H, CH_2, *J* 6.4), 2.23 (t, 2H, CH_2, *J* 7.2), 1.62 (tt, 2H, CH_2, *J* 7.2), 1.41 (s, 9H, 3CH_3); δ_C (100 MHz, DMSO-d_6): 175.14 (*C*OOH), 156.55 (*C*=O), 78.36 (*C*-Boc), 40.0 (*C*H_2), 31.9 (*C*H_2), 29.17 (*C*H_3-Boc), 25.85 (*C*H_2); *m*/*z* (ESI negative), 202.2 $[M-H]^-$.

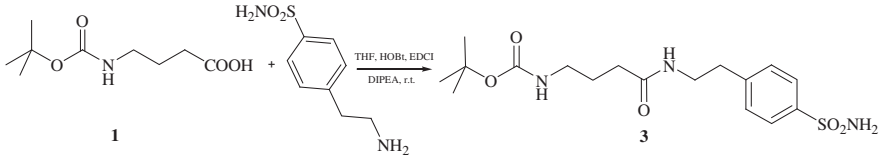
4.1.2. Synthesis of *tert*-butyl-(4-oxo-4((4-sulfamoylbenzyl)-amino)butyl)carbamate 2



4-*tert*-Butoxycarbonylamino-butyric acid **1** (1.59 g, 1.0 equiv) was suspended in dichloromethane (50 ml) and left stirring at room temperature. 4-Aminomethylbenzenesulfonamide hydrochloride (1.74 g, 1.0 equiv) and 1-hydroxybenzotriazole (1.05 g, 1.0 equiv) were then added to the reaction mixture at the same temperature followed by the additions dropwise of diisopropylethylamine (4.00 ml, 3.0 equiv) and dimethylformamide (9 ml). The reaction mixture was then cooled down to 0 °C in an ice bath and *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (1.65 g, 1.1 equiv) was added to the reaction mixture and left stirring for 65 h at room temperature. At the end of the reaction time the solution was acidified to pH 2 using a 3.0 M HCl solution and extracted with ethyl acetate. The combined organic layers were washed with 10% Citric acid, $NaHCO_3$ saturated solution, brine and then dried over anhydrous sodium sulphate, filtered, and the solvent was removed under reduced pressure to give 1.83 g of the pure product as white crystals.

tert-Butyl-(4-oxo-4((4-sulfamoylbenzyl)amino)butyl)carbamate **2**: 75% yield; δ_H (400 MHz, DMSO-d_6): 8.43 (t, 1H, N*H*, *J* 6.0, exchange with D_2O), 7.80 (d, 2H, C*H*, *J* 8.4), 7.45 (d, 2H, C*H*, *J* 8.4), 7.33 (br s, 2H, NH_2, exchange with D_2O), 6.85 (br t, 1H, N*H*, *J* 5.2, exchange with D_2O), 4.35 (d, 2H, CH_2, *J* 6.0), 2.95 (td, 2H, CH_2, *J* 6.5), 2.18 (t, 2H, CH_2, *J* 7.4), 1.66 (tt, 2H, CH_2, *J* 7.3), 1.42 (s, 9H, 3CH_3); δ_C (100 MHz, DMSO-d_6): 172.87 (C=O), 156.51 (*C*=O), 144.73 (q*C* aromatic), 143.46 (q*C* aromatic), 128.34 (2 *C*H aromatic), 126.58 (2 *C*H aromatic), 78.35 (*C*-Boc), 42.58 (*C*H_2), 33.64 (*C*H_2), 29.19 (*C*H_3-Boc), 26.67 (*C*H_2); *m*/*z* (ESI negative), 370.4 $[M-H]^-$.

4.1.3. Synthesis of [3-[2-(4-Sulfamoyl-phenyl)-ethylcarbamoyl]-propyl]-carbamic acid *tert*-butyl ester 3

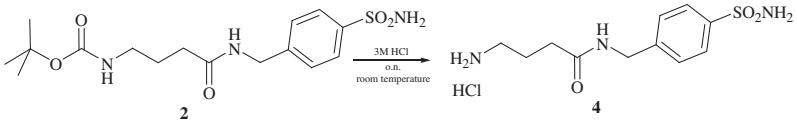


4-*tert*-Butoxycarbonylamino-butyric acid **1** (1.44 g, 1.0 equiv) was suspended in THF (70 ml) and left stirring at room temperature. 4-(2-Aminoethyl)benzenesulfonamide (1.42 g, 1.0 equiv) and 1-hydroxybenzotriazole (0.96 g, 1.0 equiv) were then added at the same temperature followed by the addition dropwise of diisopropylethylamine (2.43 ml, 2.0 equiv) and dimethylformamide (2 ml) The reaction mixture was then cooled down to 0 °C in an ice bath. *N*-(3-Dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (1.50 g, 1.1 equiv) was then added and the reaction mixture was stirred for 65 h at room temperature. At the end of the reaction time the solution was acidified to pH 2 using a 3.0 M HCl aqueous solution and then extracted with ethyl acetate. The combined organic layers were washed with 10% Citric acid, $NaHCO_3$ saturated solution, brine and then dried over anhydrous sodium sulphate, filtered, and the solvent was removed under reduced pressure to give 2.46 g of the pure product as a light brown solid.

[3-[2-(4-Sulfamoyl-phenyl)-ethylcarbamoyl]-propyl]-carbamic acid tert-butyl ester **3**: 91% yield; δ_H (400 MHz, DMSO-d_6): 7.93 (br t, 1H, N*H*, *J* 5.4, exchange with D_2O), 7.77 (d, 2H, C*H*, *J* 8.4), 7.42 (d,

2H, C*H*, *J* 8.4), 7.32 (s, 2H, NH_2, exchange with D_2O), 6.82 (br t, 1H, N*H*, exchange with D_2O), 3.32 (td, 2H, CH_2, *J* 6.4), 2.91 (td, 2H, CH_2, *J* 6.4), 2.81 (t, 2H, CH_2, *J* 7.0), 2.06 (t, 2H, CH_2, *J* 7.6), 1.60 (tt, 2H, CH_2, *J* 7.2), 1.41 (s, 9H, 3CH_3); δ_C (100 MHz, DMSO-d_6): 172.69 (*C*=O), 156.49 (*C*=O), 144.68 (q*C* aromatic), 142.94 (q*C* aromatic), 130.00 (2 *C*H aromatic), 126.58 (2 *C*H aromatic), 78.35 (*C*-Boc), 40.67 (*C*H_2), 40.50 (*C*H_2), 35.79 (*C*H_2), 33.77 (*C*H_2), 29.18 (*C*H_3-Boc), 26.69 (*C*H_2); *m*/*z* (ESI negative), 384.6 $[M-H]^-$.

4.1.4. Synthesis of 4-amino-*N*-(4-sulfamoyl-benzyl)-butyramide hydrochloride 4



A solution of *tert*-butyl-(4-oxo-4((4-sulfamoylbenzyl)amino)butyl) carbamate **2** (0.54 g, 1.0 equiv) and 3.0 M HCl (15 ml) was stirred overnight at room temperature. The solution was then dried under reduced pressure to give a yellowish oil. The oil was then washed with dichloromethane (twice) and diethylether (twice). After stirring overnight in diethylether the solvent was then removed by decantation and the powder was dried under reduced pressure to give 0.49 g of the pure product as a white solid.

4-Amino-N-(4-sulfamoyl-benzyl)-butyramide hydrochloride **4**: quantitative yield; δ_H (400 MHz, DMSO-d_6): 8.68 (br t, 1H, N*H*, *J* 5.8, exchange with D_2O), 8.06 (br s, 3H, NH_3^+, exchange with D_2O), 7.80 (d, 2H, C*H*, *J* 8.4), 7.46 (d, 2H, C*H*, *J* 8.4), 7.37 (br s, 2H, NH_2, exchange with D_2O), 4.36 (d, 2H, CH_2, *J* 6.0), 2.81 (td, 2H, CH_2, *J* 6.8), 2.32 (t, 2H, CH_2, *J* 7.2), 1.85 (tt, 2H, CH_2, *J* 7.4); δ_C (100 MHz, DMSO-d_6): 172.35 (*C*=O), 144.57 (q*C* aromatic), 143.53 (q*C* aromatic), 128.40 (2 *C*H aromatic), 126.58 (2 *C*H aromatic), 42.62 (*C*H_2), 39.27 (*C*H_2), 32.92 (*C*H_2), 24.08 (*C*H_2); *m*/*z* (ESI positive), 272.3 $[M-Cl]^+$.

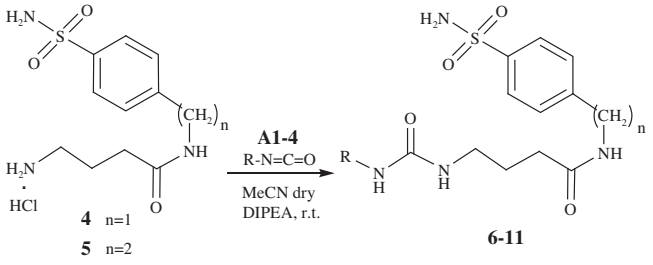
4.1.5. Synthesis of 4-amino-*N*-[2-(4-sulfamoyl-phenyl)-ethyl]-butyramide hydrochloride 5

A solution of [3-[2-(4-sulfamoyl-phenyl)-ethylcarbamoyl]-propyl]-carbamic acid *tert*-butyl ester **3** (0.5 g, 1.0 equiv) and 3.0 M HCl (10 ml) was stirred overnight at room temperature. The solution was then dried under reduced pressure to give a brownish oil. The oil was then washed with dichloromethane (twice) and diethylether (three times). After stirring overnight in diethylether the solvent was removed by decantation and the powder was dried under reduced pressure to give 0.38 g of the pure product as a light brown solid.

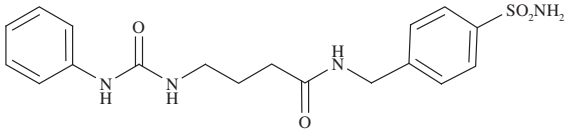
4-Amino-N-[2-(4-sulfamoyl-phenyl)-ethyl]-butyramide hydrochloride **5**: 93% yield; δ_H (400 MHz, DMSO-d_6): 8.14 (br t, 1H, N*H*, *J* 5.6, exchange with D_2O), 8.00 (br s, 3H, NH_3^+, exchange with D_2O), 7.78 (d, 2H, C*H*, *J* 8.4), 7.43 (d, 2H, C*H*, *J* 8.4), 7.35 (br s, 2H, NH_2, exchange with D_2O), 3.34 (td, 2H, CH_2, *J* 6.5), 2.83 (t, 2H, CH_2, *J* 7.2), 2.77 (m, 2H, CH_2), 2.20 (t, 2H, CH_2, *J* 7.4), 1.79 (tt, 2H, CH_2, *J* 7.4); δ_C (100 MHz, DMSO-d_6): 172.12 (*C*=O), 144.59 (q*C* aromatic), 142.97 (q*C* aromatic), 130.01 (2 *C*H aromatic), 126.59 (2 *C*H aromatic), 40.62 (*C*H_2), 39.34 (*C*H_2), 35.69 (*C*H_2), 32.97 (*C*H_2), 24.07 (*C*H_2); *m*/*z* (ESI positive), 286.5 $[M-Cl]^+$.

4.2. General procedure for the preparation of compounds 6–11

The 4-amino-*N*-(4-sulfamoyl-benzyl)-butyramide hydrochloride **4** or the 4-amino-*N*-[2-(4-sulfamoyl-phenyl)-ethyl]-butyramide hydrochloride **5** (1.0 equiv) were suspended in dry acetonitrile (10 ml) and then treated with stoichiometric amount of isocyanate **A1–4** under anhydrous conditions followed by dropwise addition of diisopropylethylamine (1.05 equiv). The mixture was stirred at room temperature for 3 h or overnight under nitrogen flow until completion (TLC monitoring). At the end of the reaction time the mixture was quenched with slush (2–3 g) and 3.0 M HCl (5 ml). The formed precipitate was then filtered off and dried in vacuo or extracted with ethyl acetate, the combined organic layers dried over anhydrous sodium sulphate, filtered and the solvent removed under reduced pressure. Further purification such as crystallization from water and washes with diethylether were required to obtain pure products. All compounds described here were characterised by spectroscopic methods.



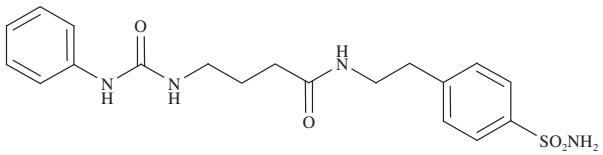
4.2.1. Synthesis of 4-(3-phenylureido)-*N*-(4-sulfamoylbenzyl)-butanamide 6



4-(3-Phenylureido)-N-(4-sulfamoylbenzyl)butanamide **6**: white solid; 70% yield; δ_H (2 atropisomers were detected in 1/0.21 ratio. Only the major one is reported herein) (400 MHz, DMSO-d_6): 8.48 (t, 1H, N*H*, *J* 5.8, exchange with D_2O), 8.45 (s, 1H, N*H*, exchange with D_2O), 7.80 (d, 2H, C*H*, *J* 8.4), 7.45 (d, 2H, C*H*, *J* 8.4), 7.42 (dd, 2H, C*H*, *J* 7.6), 7.34 (s, 2H, NH_2, exchange with D_2O), 7.24 (d, 2H, C*H*, *J* 8.0), 6.91 (dd, H, C*H*, *J* 7.2), 6.18 (t, 1H, N*H*, *J* 5.6, exchange with D_2O), 4.36 (d, 2H, CH_2, *J* 6.0), 3.13 (td, 2H, CH_2, *J* 6.4), 2.23 (t, 2H,

CH_2, *J* 7.4), 1.73 (tt, 2H, CH_2, *J* 7.2); δ_C (100 MHz, DMSO-d_6): 172.91 (*C*=O), 156.17 (*C*=O), 144.73 (q*C* aromatic), 143.47 (q*C* aromatic), 141.48 (q*C* aromatic), 129.52 (2 *C*H aromatic), 128.37 (2 *C*H aromatic), 126.59 (2 *C*H aromatic), 121.85 (1 *C*H aromatic), 118.54 (2 *C*H aromatic), 42.62 (CH_2), 39.69 (CH_2), 33.67 (CH_2), 27.03 (CH_2), *m*/*z* (ESI negative), 389.3 $[M-H]^-$.

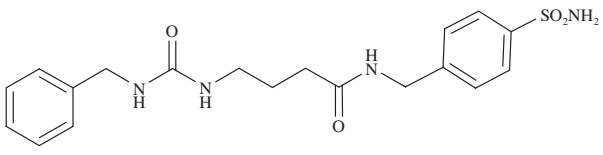
4.2.2. Synthesis of 4-(3-phenylureido)-N-(4-sulfamoylphenethyl)-butanamide 7



7

4-(3-Phenylureido)-N-(4-sulfamoylphenethyl)butanamide **7**: yellow solid; 37% yield; δ_H (400 MHz, DMSO-d_6): 8.49 (s, 1H, N*H*, exchange with D_2O), 7.98 (t, 1H, N*H*, *J* 5.4 exchange with D_2O), 7.78 (d, 2H, C*H*, *J* 8.4), 7.43 (d, 2H, C*H*, *J* 8.4), 7.42 (d, 2H, C*H*, *J* 8.4), 7.32 (s, 2H, NH_2, exchange with D_2O), 7.24 (dd, 2H, C*H*, *J* 8.0), 6.91 (dd, 1H, C*H*, *J* 7.2), 6.20 (t, 1H, N*H*, *J* 5.6, exchange with D_2O), 3.33 (td, 2H, CH_2, *J* 6.6), 3.09 (td, 2H, CH_2, *J* 6.4), 2.82 (t, 2H, CH_2, *J* 7.2), 2.12 (t, 2H, CH_2, *J* 7.4), 1.67 (tt, 2H, CH_2, *J* 7.2); δ_C (100 MHz, DMSO-d_6): 172.72 (*C*=O), 156.18 (*C*=O), 144.69 (q*C* aromatic), 142.93 (q*C* aromatic), 141.50 (q*C* aromatic), 130.01 (2 *C*H aromatic), 129.52 (2 *C*H aromatic), 126.58 (2 *C*H aromatic), 121.82 (1 *C*H aromatic), 118.50 (2 *C*H aromatic), 40.67 (CH_2), 39.63 (CH_2), 35.79 (CH_2), 33.79 (CH_2), 27.03 (CH_2); *m*/*z* (ESI positive), 427.2 $[M+Na]^+$.

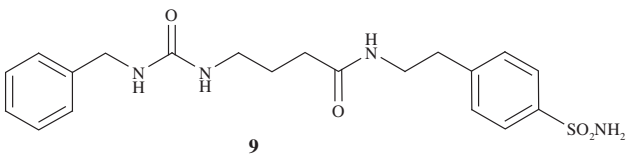
4.2.3. Synthesis of 4-(3-benzylureido)-*N*-(4-sulfamoylbenzyl)-butanamide 8



8

4-(3-Benzylureido)-N-(4-sulfamoylbenzyl)butanamide **8**: white solid; 56% yield; δ_H (400 MHz, DMSO-d_6): 8.47 (br t, 1H, N*H*, exchange with D_2O), 7.80 (d, 2H, C*H*, *J* 8.0), 7.44 (d, 2H, C*H*, *J* 8.0), 7.34 (s, 2H, NH_2, exchange with D_2O), 7.28 (m, 5H, C*H*), 6.34 (br t, 1H, N*H*, exchange with D_2O), 6.00 (br t, 1H, N*H*, exchange with D_2O), 4.35 (d, 2H, CH_2, *J* 5.6), 4.23 (d, 2H, CH_2, *J* 5.2), 3.05 (td, 2H, CH_2), 2.19 (t, 2H, CH_2, *J* 7.2), 1.67 (tt, 2H, CH_2); δ_C (100 MHz, DMSO-d_6): 173.19 (*C*=O), 159.38 (*C*=O), 144.97 (q*C* aromatic), 143.50 (q*C* aromatic), 141.90 (q*C* aromatic), 129.12 (2 *C*H aromatic), 128.36 (2 *C*H aromatic), 127.92 (2 *C*H aromatic), 127.44 (1 *C*H aromatic), 126.60 (2 *C*H aromatic), 43.83 (CH_2), 42.60 (CH_2), 39.96 (CH_2), 33.74 (CH_2), 27.31 (CH_2); *m*/*z* (ESI negative), 403.3 $[M-H]^-$.

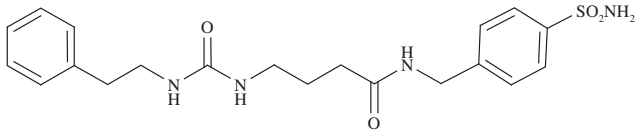
4.2.4. Synthesis of 4-(3-benzylureido)-N-(4-sulfamoylphenethyl)-butanamide 9



9

4-(3-Benzylureido)-N-(4-sulfamoylphenethyl)butanamide **9**: white solid; 66% yield; δ_H (2 atropisomers were detected in 1/0.21 ratio. Only the major one is reported herein) (400 MHz, DMSO-d_6): 7.97 (t, 1H, N*H*, *J* 5.4 exchange with D_2O), 7.77 (d, 2H, C*H*, *J* 8.4), 7.42 (d, 4H, CH, *J* 8.4), 7.33 (s, 2H, NH_2, exchange with D_2O), 7.27 (m, 5H, CH), 6.34 (t, 1H, NH, *J* 6.0, exchange with D_2O), 5.98 (t, 1H, NH, *J* 5.6, exchange with D_2O), 4.23 (d, 2H, CH_2, *J* 6.4), 3.32 (td, 2H, CH_2, *J* 6.6), 3.01 (td, 2H, CH_2, *J* 6.5), 2.81 (t, 2H, CH_2, *J* 7.2), 2.08 (t, 2H, CH_2, *J* 7.6), 1.61 (tt, 2H, CH_2, *J* 7.2); δ_C (100 MHz, DMSO-d_6): 172.78 (*C*=O), 159.01 (*C*=O), 144.68 (q*C* aromatic), 142.91 (q*C* aromatic), 141.86 (q*C* aromatic), 130.00 (2 *C*H aromatic), 129.09 (2 *C*H aromatic), 127.90 (2 *C*H aromatic), 127.42 (2 *C*H aromatic), 126.56 (1 *C*H aromatic), 43.81 (CH_2), 40.75 (CH_2), 39.97 (CH_2), 35.78 (CH_2), 33.81 (CH_2), 27.27 (CH_2); *m*/*z* (ESI negative), 417.3 $[M-H]^-$.

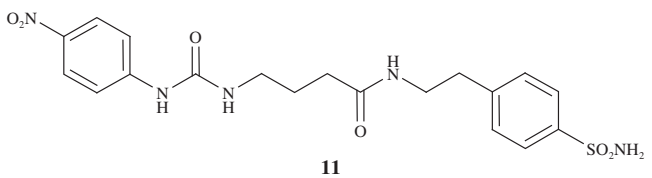
4.2.5. Synthesis of 4-(3-phenethylureido)-*N*-(4-sulfamoylbenzyl)-butanamide 10



10

4-(3-Phenethylureido)-N-(4-sulfamoylbenzyl)butanamide **10**: white solid; 58% yield; δ_H (400 MHz, DMSO-d_6): 8.47 (t, 1H, NH, *J* 5.8, exchange with D_2O), 7.80 (d, 2H, CH, *J* 8.4), 7.45 (d, 2H, CH, *J* 8.0), 7.34 (s, 2H, NH_2, exchange with D_2O), 7.31 (d, 2H, CH, *J* 7.2), 7.23 (m, 3H, CH), 5.95 (t, 1H, NH, *J* 5.6 exchange with D_2O), 5.84 (t, 1H, NH, *J* 5.6, exchange with D_2O), 4.35 (d, 2H, CH_2, *J* 6.0), 3.25 (td, 2H, CH_2, *J* 6.4), 3.01 (td, 2H, CH_2, *J* 6.5), 2.71 (t, 2H, CH_2, *J* 7.2), 2.18 (t, 2H, CH_2, *J* 7.6), 1.65 (tt, 2H, CH_2, *J* 7.2); δ_C (100 MHz, DMSO-d_6): 173.14 (*C*=O), 159.05 (*C*=O), 144.79 (q*C* aromatic), 143.47 (q*C* aromatic), 140.70 (q*C* aromatic), 129.61 (2 *C*H aromatic), 129.26 (2 *C*H aromatic), 128.40 (2 *C*H aromatic), 126.63 (1 *C*H aromatic), 126.35 (2 *C*H aromatic), 42.64 (CH_2), 41.93 (CH_2), 39.86 (CH_2), 37.12 (CH_2), 33.78 (CH_2), 27.32 (CH_2); *m*/*z* (ESI negative), 417.3 $[M-H]^-$.

4.2.6. Synthesis of 4-(3-(4-nitrophenyl)ureido)-N-(4-sulfamoyl-phenethyl)butanamide 11



11

4-(3-(4-Nitrophenyl)ureido)-N-(4-sulfamoylphenethyl)butanamide **11**: yellow solid; 36% yield; δ_H (400 MHz, DMSO-d_6): 9.32 (s, 1H, N*H*, exchange with D_2O), 8.17 (d, 2H, C*H*, *J* 9.2), 7.99 (t, 1H, N*H*, *J* 5.6, exchange with D_2O), 7.77 (d, 2H, C*H*, *J* 8.0), 7.66 (d, 2H, C*H*, *J* 9.2), 7.42 (d, 2H, C*H*, *J* 8.0), 7.33 (s, 2H, NH_2, exchange with D_2O), 6.50 (t, 1H, N*H*, *J* 5.6, exchange with D_2O), 3.32 (td, 2H, CH_2, *J* 6.4), 3.13 (td, 2H, CH_2, *J* 6.5), 2.81 (t, 2H, CH_2, *J* 7.2), 2.12 (t, 2H, CH_2, *J* 7.6), 1.70 (tt, 2H, CH_2, *J* 7.2); δ_C (100 MHz, DMSO-d_6): 172.67 (*C*=O), 155.36 (*C*=O), 148.17 (q*C* aromatic), 144.67 (q*C* aromatic), 142.93 (q*C* aromatic), 141.26 (q*C* aromatic), 130.01 (2 *C*H aromatic), 126.57 (2 *C*H aromatic), 126.05 (2 *C*H aromatic), 117.71 (2 *C*H aromatic), 40.64 (CH_2), 39.71 (CH_2), 35.77 (CH_2), 33.71 (CH_2), 26.72 (CH_2); *m*/*z* (ESI negative), 448.3 $[M-H]^-$.

4.3. CA inhibition

An Applied Photophysics stopped-flow instrument has been used for assaying the CA catalyzed CO_2 hydration activity.[17] Phenol red (at a concentration of 0.2 mM) has been used as indicator, working at the absorbance maximum of 557 nm, with 20 mM Hepes (pH 7.4) and 20 mM $NaBF_4$ (for maintaining constant the ionic strength), following the initial rates of the CA-catalyzed CO_2 hydration reaction for a period of 10–100 s. The CO_2 concentrations ranged from 1.7 to 17 mM for the determination of the kinetic parameters and inhibition constants. For each inhibitor, at least six traces of the initial 5–10% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Stock solutions of inhibitor (10 mM) were prepared in distilled–deionized water and dilutions up to 0.01 nM were done thereafter with distilled–deionized water. 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. The inhibition constants were obtained by non-linear least-squares methods using PRISM 3, whereas the kinetic parameters for the uninhibited enzymes from Lineweaver–Burk plots, as reported earlier[1–3] and represent the mean from at least three different determinations. All CAs were recombinant proteins obtained as reported earlier by these groups.[1,12,13]

Acknowledgments

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