Carbonic Anhydrase and Matrix Metalloproteinase Inhibitors: Sulfonylated Amino Acid Hydroxamates with MMP Inhibitory Properties Act as Efficient Inhibitors of CA Isozymes I, II, and IV, and *N*-Hydroxysulfonamides Inhibit Both These Zinc Enzymes[1]

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Received January 21, 2000

The 14 different carbonic anhydrase (CA, EC 4.2.1.1) isozymes as well as the 23 different matrix metalloproteinases (MMPs) isolated up to now in higher vertebrates play important physiological functions in these organisms. Unsubstituted sulfonamides act as high-affinity inhibitors for the first type of these enzymes, whereas hydroxamates strongly inhibit the latter ones. Since the active site geometry around the zinc ion in these two types of metalloenzymes is rather similar, we tested whether sulfonylated amino acid hydroxamates of the type RSO_2-NX-AA-CONHOH (X = H, benzyl, substituted benzyl; AA = amino acid moiety, such as Gly, Ala, Val, Leu) with well-known inhibitory properties against MMPs and *Clostridium histolyticum* collagenase (ChC, another zinc enzyme related to the MMPs) might also act as CA inhibitors. We also investigated whether *N*-hydroxysulfonamides of the type RSO_2NHOH (which are effective CA inhibitors) inhibit MMPs and ChC. Here we report several potent sulfonylated amino acid hydroxamate CA inhibitors (with inhibition constants in the range of 5−40 nM, against the human isozymes hCA I and hCA II, and 10−50 nM, against the bovine isozyme bCA IV), as well as preliminary SAR for this new class of non-sulfonamide CA inhibitors. Some *N*-hydroxysulfonamides also showed inhibitory properties (in the micromolar range) against MMP-1, MMP-2, MMP-8, MMP-9, and ChC. Thus, the SO_2NHOH group is a new zinc-binding function for the design of MMP inhibitors. Both CA as well as MMPs are involved, among others, in carcinogenesis and tumor invasion processes. On the basis of these findings, we suggest that the mechanism of antitumor action with some hydroxamate inhibitors might also involve inhibition of some CA isozymes (such as CA IX, CA XII, and CA XIV) present only in tumor cell membranes, in addition to collagenases/gelatinases of the MMP type. Our data also suggest that it should be possible to develop dual enzyme inhibitors that would strongly inhibit both these metalloenzymes, CAs and MMPs, based on the nature of the R, AA, and X moieties in the above formula. Compact X (such as H) and AA (such as Gly) moieties favor CA over MMP inhibition, whereas bulkier X (benzyl, substituted benzyl, etc.) and AA (such as Val, Leu) moieties and substituted-aryl R groups are advantageous for obtaining potent MMP and ChC inhibitors, which show lower affinity for CA.

Introduction

The ubiquitous enzyme carbonic anhydrase (CA, EC 4.2.1.1) is present in Archaea, prokaryotes, and eukaryotes as three different families of enzymes:[2,3] the α-CA (mainly in vertebrates and in some green plants), *β*-CA (mainly in bacteria, algae, and green plants) and *γ*-CA (only in Archaea) families, respectively.[2,3] In higher vertebrates, including humans, 14 different CA isozymes have been described up to now[2] that are involved in crucial physiological processes connected with respiration and transport of CO_2/bicarbonate between metabolizing tissues and the lungs, pH homeostasis, electrolyte secretion in a variety of tissues/organs, and biosynthetic reactions, such as lipogenesis, gluconeogenesis, and ureagenesis, among others.[2−7] Some of these isozymes are cytosolic (such as CA I, CA II, CA III, and CA VII); others are membrane-bound (CA IV, CA IX, CA XII, and CA XIV). CA V is present only in mitochondria, and CA VI is secreted in saliva, whereas several acatalytic forms are also known (CA VIII, CA X, and CA XI).[2−15]

In addition to the physiological reaction, the reversible hydration of carbon dioxide to bicarbonate, CAs also catalyze a variety of other reactions,[16−25] such as aldehyde hydration[16] and hydrolysis of carboxylic acid esters,[17,18] whereas esters of sulfonic[19] or phosphoric[20] acids also seem to act as substrates of these enzymes. Other hydrolytic reactions in which CAs may participate, but which have not thoroughly been studied from the mechanistic point of view, include hydrolyses of diverse halogeno derivatives such as 2,4-dinitrofluorobenzene,[21] benzyloxycarbonyl chloride,[22] or sulfonyl chlorides.[23] Recently, the cyanate and cyanamide hydration reactions catalyzed by several CA isozymes, leading to suicide inhibitors of this enzyme, have also been investigated spectroscopically (using Co(II)-substituted enzyme), kinetically, and by X-ray crystallography by our group.[24,25] On the other hand, CAs do not possess peptidase activity,[26,27] which in turn is the only reaction catalyzed by the proteases of the matrix

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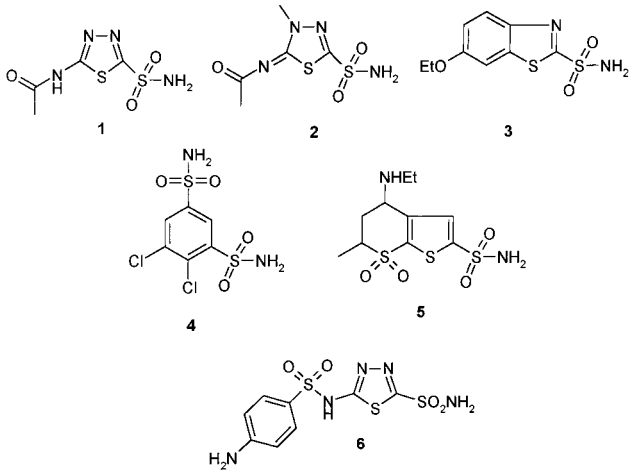
10.1021/jm000027t CCC: $19.00
Published on Web 09/13/2000

metalloproteinase (MMP) family with very great efficiency.[26−32]

Specific inhibitors of both these types of zinc enzymes are well-known, and some of them were clinically used for more than 45 years (the sulfonamide CA inhibitors). Inhibition of CAs by aromatic/heterocyclic sulfonamides such as **1**−**5** has been successfully used in the treatment of a variety of diseases such as glaucoma,[4,33] epilepsy,[34] congestive heart failure,[5] mountain sickness,[35] and gastric and duodenal ulcers[36] or as diuretic agents.[37] MMPs on the other hand became targets for drug design only recently, but several drugs of this type might reach clinics soon, as anticancer or antiarthritis agents among others.[27,30−32]



Recently this[38] and other groups also showed[39] that some new types of sulfonamide CA inhibitors act as efficient tumor cell growth inhibitors in vitro and in vivo, by a mechanism of action that might involve acidification of the intratumoral environment ensued after CA inhibition[38,39] or 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.[39] On the other hand, some new CA isozymes, such as CA IX,[9] CA XII,[12,15] and CA XIV,[13] were predominantly found to be present only in tumor cells. These and other more classical isozymes, such as CA I, CA II, and CA IV,[40,41] were also shown to be present and actively involved in other types of proliferative diseases/processes, such as von Hippel-Lindau renal tumors,[15] progressive polycystic kidney disease,[40] acinar-ductal carcinomas of the pancreas,[41] autoimmune or idiopathic chronic pancreatitis,[41] and apoptosis in some human pancreatic cancer cells.[41] Since many hydroxamate MMP inhibitors were developed just as possible antitumor/antimetastatic agents,[27,30−33] and considering the fact that all inhibitors mentioned above (of CAs or of MMPs) bind to the metal ion within the active site cavity, it appeared of interest to further explore the connections between CA and MMP inhibitors, with the eventual evidence of a "cross-reactivity" between them. Here we report the observation that several potent MMP inhibitors of the sulfonylated amino acid hydroxamate type also act as efficient CA inhibitors (with inhibition constants in the range of 7−45 nM, against the human isozymes hCA I and hCA II, and 10−50 nM, against the bovine isozyme bCA IV), as well as preliminary SAR for this new class of non-sulfonamide CA inhibitors. Some *N*-hydroxysulfonamides investigated previously as CA inhibitors also showed inhibitory properties (in the micromolar range) against MMP-1, MMP-2, MMP-8, MMP-9, and a bacterial collagenase (ChC) isolated from *Clostridium histolyticum*.

Results

Synthesis. The majority of the *N*-hydroxysulfonamides[42] and some of the sulfonylated amino acid hydroxamates[43,44] investigated here have previously been reported by this group. Some of the compounds investigated in this paper are new (such as **7**−**9**, **13**−**15**, **19**−**21**, **25**−**33**, **69**, **71**−**80**). They were synthesized by the same methods used for the preparation of amino acid hydroxamate/*N*-hydroxysulfonamide derivatives reported in previous contributions from our group.[42−44]

CA Inhibitory Activity. Inhibition data against three CA isozymes, hCA I, hCA II, and bCA IV, with the sulfonylated amino acid hydroxamates **7**−**45** and the *N*-hydroxysulfonamides **46**−**80** are shown in Tables 1 and 2. The esterase activity of CA isozymes against 4-nitrophenyl acetate as substrate has been used in this assay[45] (see Experimental Section for details).

MMP and ChC Inhibitory Activity. Inhibition data against four MMPs (MMP-1, MMP-2, MMP-8, and MMP-9) and type II ChC with the compounds investigated in the present paper (**7**−**80**) are shown in Tables 1 and 2. The thioester (AcProLeuGly-S-LeuLeuGlyOEt) spectrophotometric method of Powers and Kam,[46a] modified by Johnson et al.,[46b] was used for assessing the catalytic activity and inhibition of the four MMPs mentioned above, whereas the amidolytic spectrophotometric method with FALGPA as substrate was used for the ChC inhibition assays.[47]

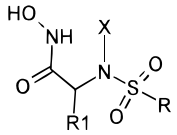
Spectroscopic Studies on Co(II)-Substituted Enzymes. Electronic spectroscopic data for Co(II)-substituted hCA II and ChC and their adducts with standard and new inhibitors of the type reported here are shown in Table 3.

Discussion

Chemistry. CAs and MMPs possess very similar metal coordination spheres within their catalytic sites, consisting of a Zn(II) ion coordinated by three histidines, with the fourth ligand being a water molecule/hydroxide ion, which is the nucleophile intervening in the catalytic cycle of both enzymes (Figure 1).[4,26,27,48,49]

The main structural difference between these two types of enzymes regards the residues with which the zinc-bound water molecule/hydroxide ion interacts: in CAs, the non-protein zinc ligand forms a hydrogen bond with the hydroxyl moiety of Thr 199 (hCA II numbering), which in turn is hydrogen-bonded to the carboxylate of Glu 106, leading thus to a dramatic enhance of nucleophilicity of the water molecule/hydroxide ion.[4,25−27] In the case of MMPs, the zinc-bound water molecule interacts with the carboxylate moiety of a conserved glutamate residue (Glu 198 in MMP-8), probably forming two hydrogen bonds with it.[27,48,49] Thus, a very effective nucleophile is formed again, which will attack the amide scissile bond of the peptide substrate. The principal difference between the enzymatic mechanisms of CAs and MMPs consists of the fact that the nucleophilic adduct formed after the attack of the zinc-bound

Table 1. Inhibition of MMPs, ChC, and CAs with the Hydroxamates **7**−**45**



7 - 45

				K_i[a] (nM)							
no.	R1	R	X	MMP-1[b]	MMP-2[b]	MMP-8[b]	MMP-9[b]	ChC[c]	hCA I[d]	hCA II[d]	bCA IV[d]
7	H	*n*-C_4F_9	H	>200	75	130	125	80	18	15	16
8	H	C_6F_5	H	145	44	125	100	54	7	8	10
9	H	4-MeO-C_6H_4	H	>200	110	155	143	120	30	32	29
10	H	*n*-C_4F_9	$C_6H_5CH_2$	30	3.9	5.3	5	13	105	85	100
11	H	C_6F_5	$C_6H_5CH_2$	7	1.5	1.1	1.2	6	90	36	42
12	H	4-MeO-C_6H_4	$C_6H_5CH_2$	60	18	31	42	27	>200	120	145
13	Me	*n*-C_4F_9	H	>200	69	118	121	79	21	16	17
14	Me	C_6F_5	H	150	40	116	96	45	7	8	10
15	Me	4-MeO-C_6H_4	H	>200	87	125	137	130	32	35	30
16	Me	*n*-C_4F_9	$C_6H_5CH_2$	26	3.2	4.9	4.3	12	121	92	107
17	Me	C_6F_5	$C_6H_5CH_2$	7	0.9	1.1	1.4	6	84	38	43
18	Me	4-MeO-C_6H_4	$C_6H_5CH_2$	58	15	19	35	20	195	120	136
19	^{i}Pr	*n*-C_4F_9	H	>200	66	111	120	78	29	15	20
20	^{i}Pr	C_6F_5	H	139	41	104	89	40	8	11	13
21	^{i}Pr	4-MeO-C_6H_4	H	>200	82	126	126	103	33	39	38
22	^{i}Pr	*n*-C_4F_9	$C_6H_5CH_2$	21	2.4	4.2	4.3	10	140	108	124
23	^{i}Pr	C_6F_5	$C_6H_5CH_2$	7	0.8	1.0	1.2	5	88	45	48
24	^{i}Pr	4-MeO-C_6H_4	$C_6H_5CH_2$	43	11	13	27	17	>200	>200	185
25	^{i}Bu	*n*-C_4F_9	H	>200	62	108	36	69	36	18	30
26	^{i}Bu	C_6F_5	H	155	39	101	122	38	10	11	19
27	^{i}Bu	4-MeO-C_6H_4	H	>200	84	123	78	95	50	5	39
28	^{i}Bu	*n*-C_4F_9	$C_6H_5CH_2$	16	1.9	3.3	4.0	8	180	116	139
29	^{i}Bu	C_6F_5	$C_6H_5CH_2$	6	0.8	0.6	1.1	5	100	56	75
30	^{i}Bu	4-MeO-C_6H_4	$C_6H_5CH_2$	44	10	9	1.3	13	>200	>200	190
31	H	*n*-C_4F_9	2-$O_2NC_6H_4CH_2$	25	3.7	5.5	4.6	13	>200	100	120
32	H	C_6F_5	2-$O_2NC_6H_4CH_2$	6	1.4	1.0	1.3	6	127	74	49
33	H	4-MeO-C_6H_4	2-$O_2NC_6H_4CH_2$	54	15	27	39	24	>200	170	140
34[e]	H	*n*-C_4F_9	4-$O_2NC_6H_4CH_2$	62	1.5	2.4	2.0	12	>200	118	136
35[e]	H	C_6F_5	4-$O_2NC_6H_4CH_2$	3	0.7	0.1	0.6	5	150	107	130
36[e]	H	4-MeO-C_6H_4	4-$O_2NC_6H_4CH_2$	28	18	21	31	20	>200	>200	>200
37[f]	Me	*n*-C_4F_9	2-$O_2NC_6H_4CH_2$	24	2.9	5.1	4.4	10	>200	>200	>200
38[f]	Me	C_6F_5	2-$O_2NC_6H_4CH_2$	7	0.8	1.1	1.0	5	>200	160	>200
39[f]	Me	4-MeO-C_6H_4	2-$O_2NC_6H_4CH_2$	39	13	20	24	19	>200	>200	>200
40[g]	Me	*n*-C_4F_9	4-$O_2NC_6H_4CH_2$	60	1.4	2.3	1.5	11	>200	>200	>200
41[g]	Me	C_6F_5	4-$O_2NC_6H_4CH_2$	4	0.7	0.3	0.6	5	>200	>200	>200
42[g]	Me	4-MeO-C_6H_4	4-$O_2NC_6H_4CH_2$	25	15	18	28	21	>200	>200	>200
43[h]	Me	*n*-C_4F_9	2-$ClC_6H_4CH_2$	37	3.7	6.4	5.0	11	>200	>200	>200
44[h]	Me	C_6F_5	2-$ClC_6H_4CH_2$	10	1.5	1.3	1.7	5	>200	>200	>200
45[h]	Me	4-MeO-C_6H_4	2-$ClC_6H_4CH_2$	53	12	27	31	22	>200	>200	>200

[a] K_i values were obtained from Easson−Stedman[56] plots using a linear regression program, from at least three different assays. Standard errors were of 5−10%. [b] With the thioester substrate Ac-ProLeuGly-S-LeuLeuGlyOEt, spectrophotometrically.[46] [c] With FALGPA as substrate, spectrophotometrically.[47] [d] With 4-NPA as substrate, by the esterase method, spectrophotometrically.[45] [e] MMP and ChC inhibition (but not CA inhibition) data from ref 43c. [f] ChC inhibition (but not MMP and CA inhibition) data from ref 58. [g] ChC inhibition (but not MMP and CA inhibition) data from ref 43b. [h] ChC inhibition (but not MMP and CA inhibition) data from ref 59.

nucleophile to the substrate is the reaction product in the case of CAs (the bicarbonate ion), whereas the nucleophilic adduct is only a reaction intermediate in the case of the MMPs (and also ChC).[26,27] This is also of crucial importance for the interaction of these enzymes with their inhibitors.

Inhibition of both CAs as well as MMPs is correlated with the coordination of the inhibitor molecule (in neutral or ionized state) to the catalytic metal ion, with or without substitution of the metal-bound water molecule.[4,26,27,30] Thus, CA and MMP inhibitors (abbreviated as CAIs and MMPIs, respectively) must contain a zinc-binding function attached to a scaffold that will interact with other binding regions of the enzymes.[4,26,27,30] In the case of CAIs, unsubstituted aromatic/heterocyclic sulfonamides[4,5] as well as *N*-hydroxysulfonamides[42] proved to be very effective inhibitors, with affinities in the low nanomolar range for isozymes such as CA I, CA II, CA IV, etc. These derivatives bind monodentately, as anions (RSO_2NH^-) to the Zn(II) ion within the CA active site, interacting also with several other active site residues, by means of hydrogen bonds or hydrophobic interactions.[50] Residues situated at the entrance of the active site, such as the histidine cluster comprising residues His 64, His 3, His 4, and His 10, among others, seem to be critically important for the formation of strong enzyme−inhibitor adducts.[4,33,50] In fact, it has been observed that CAIs possessing elongated molecules, able to interact with amino acid residues situated at the edge of the active site entrance (and obviously with the zinc ion, as mentioned above), are among the most efficient ones (such as, for example, aminobenzolamide **6**), and this has also been explained theoretically in several QSAR studies of one of our groups.[51]

Table 2. Inhibition of MMPs, ChC, and CAs with the Hydroxysulfonamides **46**−**80**

RSO_2NHOH
46-80

		K_i[a] (μM)							
no.	R	MMP-1[b]	MMP-2[b]	MMP-8[b]	MMP-9[b]	ChC[c]	hCA I[d]	hCA II[d]	bCA IV[d]
46	Me	>100	>100	>100	>100	>100	>100	4.7	56
47[e]	CF_3	>100	>100	>100	>100	>100	20	0.03	0.016
48	CCl_3	>100	>100	>100	>100	>100	23	0.15	0.21
49	n-C_4F_9	74	19	25	21	62	1.8	0.015	0.027
50	n-C_8F_{17}	70	14	23	21	55	1.6	0.009	0.021
51[e]	Me_2N	>100	>100	>100	>100	>100	12	0.040	0.29
52[e]	$PhCH_2$	95	73	76	83	80	19	0.19	0.46
53	Ph	79	70	74	78	83	18	0.026	0.074
54[e]	p-F-C_6H_4	74	72	75	73	79	15	0.019	0.090
55[e]	p-Cl-C_6H_4	71	70	70	76	80	27	0.021	0.120
56[e]	p-Br-C_6H_4	71	65	69	66	72	38	0.018	0.039
57[e]	p-I-C_6H_4	66	72	71	69	70	15	0.013	0.045
58[e]	p-CH_3-C_6H_4	65	77	64	60	88	51	0.070	0.127
59[e]	p-O_2N-C_6H_4	42	21	15	14	36	57	0.009	0.070
60[e]	m-O_2N-C_6H_4	37	23	16	13	32	17	0.005	0.024
61[e]	o-O_2N-C_6H_4	59	38	24	23	62	15	0.005	0.013
62[e]	3-Cl-4-O_2N-C_6H_3	30	14	16	13	29	17	0.004	0.012
63[e]	p-AcNH-C_6H_4	29	9	12	10	18	40	0.029	0.040
64[e]	p-H_2N-C_6H_4	34	15	21	24	50	62	0.037	0.055
65	4-MeO-3-H_2N-C_6H_3	30	11	10	9	13	41	0.044	0.136
66[e]	p-CH_3O-C_6H_4	33	14	16	12	20	68	0.051	0.110
67[e]	2,4,6-$(CH_3)_3$-C_6H_2	40	21	36	34	39	72	0.062	0.156
68[e]	C_6F_5	0.4	0.9	1.0	1.3	0.5	30	0.0008	0.013
69	3-CF_3-C_6H_4	0.9	0.12	1.5	1.7	0.8	33	0.0011	0.016
70[e]	o-HOOC-C_6H_4	0.08	0.09	0.12	0.10	7	25	0.0009	0.012

[a] K_i values were obtained from Easson−Stedman[56] plots using a linear regression program, from at least three different assays. Standard errors were of 5−10%. [b] With the thioester substrate Ac-ProLeuGly-S-LeuLeuGlyOEt, spectrophotometrically.[46] [c] With FALGPA as substrate, spectrophotometrically.[47] [d] With 4-NPA as substrate, by the esterase method, spectrophotometrically.[45] [e] CA inhibition (but not MMP and ChC inhibition) data from ref 42.

Table 3. Spectral Data (in the range 400−750 nm) for Adducts of Co(II)-hCA II and Co(II)-ChC with Standard and New Inhibitors Reported Here[a]

adduct	pH	band position (nm) and molar absorptivity (M^{-1} cm^{-1})
Co(II)-hCA II	8.0	520 (280); 550 (380); 616.5 (280); 640 (260)
+acetazolamide **1**	8.0	518 (390); 549 (220); 574 (530); 595 sh (500)
+thiocyanate	8.1	465 (100); 529 sh (90); 571 (100); 689 (9)
+nitrate	7.5	470 (100); 515 (80); 555 (110); 709 (9)
+acetate	7.5	470 (100); 515 sh (80); 555 (110); 709 (9)
+benzoate	7.5	480 sh (110); 507 sh (170); 555 (110); 709 (9)
+**8**	7.5	475 (110); 515 sh (80); 555 (115); 710 (9)
+**19**	7.5	470 (110); 515 sh (90); 555 (110); 710 (9)
+**50**	8.0	518 (320); 550 (300); 574 (380); 600 sh (480)
+**60**	8.0	519 (370); 550 sh (380); 575 (360); 597 sh (400)
Co(II)-ChC	6.5	530 sh (135); 585 (180)
+**8**	6.5	505 sh (85); 563 (120); 598 (15)
+**11**	6.5	505 sh (85); 562 (115); 597 (13)
+**23**	6.5	505 sh (85); 563 (117); 597 (15)
+**35**	6.5	503 sh (80); 562 (112); 598 (15)
+**65**	6.5	501 sh (78); 563 (105); 598 (11)
+**69**	6.5	501 sh (79); 563 (109); 598 (11)

[a] Enzyme concentrations were in the range 0.1−0.4 nM, at the pH values specified in each case. Inhibitor concentrations were in the range of 0.1−2 mM.

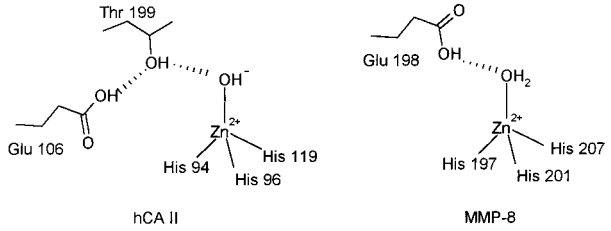


Figure 1. Active site coordination of the Zn(II) ion in human carbonic anhydrase isozyme II (hCA II) and human collagenase 2 (MMP-8). The non-protein zinc ligand of hCA II may be a hydroxide ion (as shown above) or a water molecule, depending on the pH.

Depending on the zinc-binding function contained in their molecule, MMPIs belong to several chemical classes, such as the carboxylates, the hydroxamates, the thiols, the phosphorus-based ligands, or the sulfodiimines, among others.[27,30] The strongest class of MMPIs is constituted by the hydroxamates.[27,30] The interaction of the catalytic domain of several MMPs with some inhibitors has been recently investigated by means of X-ray crystallography (Figure 2).[32,48,49,52]

As seen in Figure 2, hydroxamates bind bidentately to the catalytic Zn(II) ion of the enzyme, which acquires a distorted trigonal-bipyramidal geometry in this way.[48,49,52] The hydroxamate anion forms a short and strong hydrogen bond with the carboxylate moiety of Glu 219, which is oriented toward the unprimed binding regions, whereas the NH hydroxamate participates in a hydrogen bond with the carbonyl oxygen of Ala 182. Thus, several strong interactions are achieved at the zinc site, without any significant unfavorable contacts.

Recently, Christianson's group investigated two simple hydroxamates (methyl and trifluoromethyl hydroxamates) as possible CAIs and reported the X-ray structure of such an adduct (hCA II−$CF_3CONHOH$).[53] This compound binds to hCA II with an affinity of 3.8 μM,

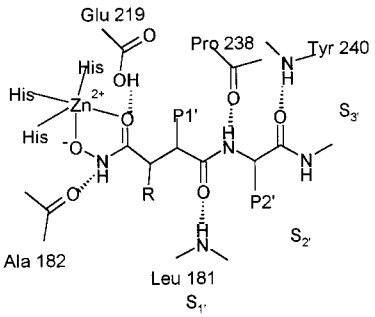


Figure 2. Schematic representation for the binding of a succinate hydroxamate inhibitor to MMP-7, as determined by X-ray crystallography.[48] The Zn(II)−ligand and hydrogen bond interactions in the enzyme−inhibitor adduct are evidenced (adapted and modified from ref 48).

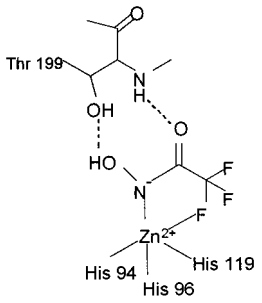
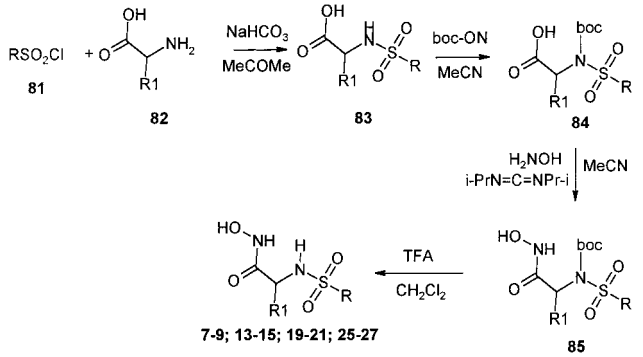


Figure 3. Schematic binding of trifluoromethyl hydroxamate to hCA II (adapted from ref 53).

but its interaction with the Zn(II) ion of CA active site is very different from that of the classical sulfonamide inhibitors. Thus, the ionized nitrogen atom of the hydroxamate moiety is directly coordinated to Zn(II), whereas a fluorine atom of the trifluoromethyl moiety also participates in the interaction with the metal ion (Figure 3). In addition, hydrogen bonds between the hydroxamate OH and the active site residue Thr 199 were also evidenced,[53] which further stabilize the E−I adduct (Figure 3).

On the basis of all these data presented above, we decided to test sulfonylated amino acid hydroxamates (recently reported to act as strong MMPIs)[43,44,54] as potential CAIs. The following types of compounds were included in this study: (i) Sulfonylated amino acid hydroxamates possessing an unsubstituted RSO_2NH-amino acyl moiety. The amino acid hydroxamates included in the study were the Gly, Ala, Val, and Leu derivatives (Table 1). (ii) Sulfonylated amino acid hydroxamates possessing a substituted RSO_2NX-amino acyl moiety, where X is generally a benzyl or 2- or 4-substituted-benzyl group (the same amino acid hydroxamates as above were included in the study, Table 1). For all these types of compounds, three different examples have been used (R moieties) from the large series of available aliphatic, aromatic, and heterocyclic derivatives reported previously by our group.[43,44] They included the perfluorobutyl, pentafluorophenyl, and 4-methoxyphenylsulfonyl moieties and were chosen in such a way as to include a very potent, a slightly weaker, and an even weaker MMPI. Anyhow, all three groups incorporated in amino acid hydroxamates generally led to potent MMPIs, with affinities (for the most active derivatives) in the (low) nanomolar range (5−15 nM) for the different MMPs and ChC.[43,44] (iii) Simple

Scheme 1



aliphatic and aromatic *N*-hydroxysulfonamides of the type **46**−**53**, as well as aryl/hetaryl mono-/di-/tri-/pentasubstituted derivatives, possessing a large variety of substituents at the aromatic/heterocyclic moiety, of types **54**−**79** (Table 2).

Synthetic variants used for the preparation of the previously reported sulfonylated amino acid hydroxamates[43,44] have been modified for obtaining the new compounds reported here (Scheme 1).

Reaction of sulfonyl chlorides **81** with amino acids **82** led to the alkyl/arylsulfonyl amino acids **83**,[43,44] which were then protected at the NH moiety by the *tert*-butyloxycarbonyl (Boc) group (with Boc-ON, 2-*tert*-butoxycarbonyloxyimino-2-phenylacetonitrile).[57] In several cases (for Gly, Ala, and Val) the reaction procedure was the one described above, whereas in other cases (Leu) better yields were obtained when the sulfonyl chlorides were directly reacted with the Boc-protected amino acid. This procedure could anyhow be used successfully for the preparation of the Gly, Ala, and Val derivatives too. The Boc-protected derivatives **84** were converted to the corresponding hydroxamic acids in the standard manner, with hydroxylamine (or *O*-Boc-hydroxylamine) and carbodiimides,[43,44] leading to derivatives **85**, which were deprotected (eventually both at the NH and OH moieties) with TFA. The use of the *O*-Boc-hydroxylamine (*t*-Bu-OCO-ONH_2) did not show any significant advantage over the simple H_2NOH for the synthesis of these hydroxamic acids. Derivatives **7**−**9**, **13**−**15**, **19**−**21**, and **25**−**27** were obtained in this way.

Reaction of sulfonyl halides with hydroxylamine afforded the new *N*-hydroxysulfonamides **69** and **71**−**80**, by the method previously reported by our group.[42]

CA, MMP, and ChC Inhibition. The inhibition data with compounds **7**−**80**, presented in Tables 1 and 2, led to the following observations: (i) Sulfonyl amino acyl hydroxamates possessing moieties of the type RSO_2NH-amino acyl (such as **7**−**9**, **13**−**15**, **19**−**21**, and **25**−**27**) generally act as efficient CA inhibitors and are relatively weak MMP and ChC inhibitors. Thus, for CAs, these inhibitors generally showed affinities in the range of 7−50 nM (hCA I);, 8−45 nM (hCA II), and 10−40 nM (bCA IV), whereas for the different MMPs investigated here and ChC, their affinities were in the range of 40−>200 nM. For the three types of investigated derivatives, the most active were the pentafluorophenylsulfonyl derivatives, followed by the corresponding perfluorobutyl ones, whereas the least active were the corresponding 4-methoxyphenyl-substituted compounds. For CA inhibition, best activity was observed for the Gly derivatives, followed by the corresponding Ala deriva-

tives, which in turn were more active than the corresponding Val and Leu derivatives. Just the opposite was generally true for MMP and ChC inhibition, with the bulkier Val and Leu derivatives generally more inhibitory than the corresponding Ala and Gly derivatives (Table 1). (ii) Sulfonyl amino acyl hydroxamates possessing RSO_2N(benzyl/substituted-benzyl)-amino acyl moieties (such as **10**−**12**, **16**−**18**, **22**−**241**, and **28**−**45**) were weak or very weak CA inhibitors but showed excellent MMP and ChC inhibitory properties. Thus, these compounds were generally 4−8 times weaker CA inhibitors as compared to the corresponding unsubstituted compounds mentioned above, whereas their affinities for MMPs were very much enhanced as compared to those of the corresponding unsubstituted compounds. It was in fact reported that the benzyl moiety of this type of hydroxamate inhibitors fits well within the $S_{2'}$ site of the protease, contributing substantially to the formation of strong E−I adducts.[30,32,49] Obviously the different MMPs possess quite diverse affinities for these derivatives, with important differences between the deep pocket (MMP-2, MMP-8, and MMP-9) and the short pocket enzymes (MMP-1).[30,32] Thus, as already shown previously by us for some structurally related derivatives,[43c] the deep pocket enzymes MMP-2, MMP-8, and MMP-9 are much more susceptible to be inhibited by this class of hydroxamates (K_i's in the range of 0.6−20 nM) than collagenase 1, MMP-1 (K_i's in the range of 7−60 nM). Again the pentafluorophenylsulfonyl derivatives were the most active inhibitors, followed by the corresponding perfluorobutyl ones, whereas the least active were the corresponding 4-methoxyphenyl-substituted compounds. The Leu derivatives were generally more active than the corresponding Val derivatives, which in turn were more inhibitory than the Ala and Gly derivatives. (iii) Further substitution (with nitro or chloro moieties, in position 2 or 4) of the $P_{2'}$ benzyl moiety, such as in compounds **31**−**45,** led to a slight enhancement of the MMP inhibitory properties, to an enhancement of the ChC inhibitory effects, and to a drastic reduction of the CA inhibitory properties of the corresponding compounds (Table 1). (iv) *N*-Hydroxysulfonamides **46**−**80** generally act as effective CAIs, with affinities in the low nanomolar range (against isozyme II) for the perhalogenoalkyl/aryl (**49, 50, 68, 73**), the nitroaryl-substituted (**59**−**62**), or the heterocyclic (**79**) compounds. Other substitution patterns of the R moiety generally led to less effective CAIs (affinities in the range of 13−50 μM for hCA I, 40−150 nM for hCA II, and 100−300 nM for bCA IV) (Table 2). (v) *N*-Hydroxysulfonamides **46**−**80** generally act as moderately weak MMP and ChC inhibitors (affinities in the 5−95 μM) with several important exceptions. Thus, *N*-hydroxyperfluorophenylsulfonamide (**68**) or the 2-hydroxy-3,5-dichlorophenyl-substituted derivative **74** showed an enhanced affinity against all the investigated MMPs (with MMP-1 and ChC more susceptible than MMP-2, MMP-8, and MMP-9), whereas the best MMPIs in the entire series (compounds **70** and **73**) arrived at affinities of around 50−120 nM against the diverse MMPs investigated here. It should be noted that both these compounds possess an *o*-carboxyl group in the neighborhood of the SO_2NHOH one, which makes probable the participation of both these functionalities in the interaction with the catalytic zinc ion of the MMPs and ChC. On the other hand, the *m*-carboxy- and *p*-carboxyphenyl-substituted derivatives **71** and **72** showed at least a 100-fold decreased affinity to the investigated MMPs, as compared to the *ortho*-substituted isomer **70** (Table 2).

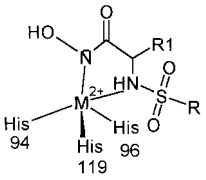


Figure 4. Proposed binding of a sulfonylated amino acid hydroxamate (as monoanion) to the metal ion within the active site of CA (M = Zn(II) for the native enzyme or Co(II) for the cobalt-substituted one).

The data of the two tables prove that potent CAIs can be obtained from the class of investigated sulfonylated amino acid hydroxamates. Although it was noted that in addition to MMPs, hydroxamates also inhibit other metalloproteinases, such as leucine aminopeptidase, neprylysin, thermolysin,[60a] and tumor necrosis factor-α,[60b] among others, affinities as high (in the nanomolar range) were not evidenced up to now. Thus, our results are quite promising for the eventual design of novel types of potent CAIs or of compounds with a dual activity, as both CAIs and MMPIs. It should be also noted that the *N*-hydroxysulfonamides were not optimized as MMPIs, since in addition to the new zinc-binding function reported here for the first time (SO_2-NHOH), modifications of moieties that bind in the $S_{1'}$, $S_{2'}$, and/or eventually $S_{3'}$ sites of the enzyme have not been performed. It is thus envisageable that potent MMPIs can be obtained incorporating this new zinc-binding function and peptidomimetic scaffolds that should fit well in the primed binding sites of these enzymes. Work is in progress in our laboratory for the design of such compounds. All these data also seem to validate our hypothesis that the new class of CAIs reported here binds to the Zn(II) ion of the enzyme as shown in Figure 4.

Spectroscopic Studies on Co(II)-Substituted Enzymes. To monitor the possible interaction of the new inhibitors reported here with the active site of these enzymes, the electronic spectra of cobalt(II)-substituted hCA II and ChC and of their adducts with standard and hydroxamate/*N*-hydroxysulfonamide inhibitors were recorded. The electronic spectral data of Table 3 indicate the folowing information. Sulfonamides, such acetazolamide **1**, bind to the Co(II) ion within the CA active site giving rise to a pseudotetrahedral geometry of the metal ion.[69] Such adducts are characterized by intense spectra with molar absorbances above 300 M^{-1} cm^{-1}.[69,70] The four absorption maxima in the spectrum of the pure enzyme (at 520, 550, 616.5, and 640 nm, respectively) undergo notable changes when inhibitor is coordinated to the metal ion. Thus, especially the last two maxima are changed dramatically after complexation, colapsing into a unique, broad maximum centered at 574−575 nm and a shoulder at 595−600 nm. The tetrahedral geometry of such E−I adducts has been confirmed by X-ray crystallographic data for some of these complexes (of the native or metal-substituted enzymes).[50] Co(II) is pen-

tacoordinated in the adducts with thiocyanate,[71a] nitrate,[71b] acetate, or benzoate, as shown in the classical studies of Bertini's group.[70,72] These spectra are characterized by molar absorbances under 150 M^{-1} cm^{-1} [69,70] and a different pattern of the four absorption maxima: thus, a maximum appears under 490 nm (generally at 465−480 nm), whereas the two strong maxima in the spectrum of pure Co(II)-hCA II, at 550 and 616.5 nm, appear as a weak band, with the absorption maximum at 555−575 nm. Additionally, another maximum at 689−709 nm is seen in the electronic spectra of pentacoordinated Co(II) of such E−I adducts. The pentacoordination of the metal ion in some of these complexes has been then confirmed by the report of the X-ray structure for some of these adducts: hCA II thiocyanate by Liljas' group[71a] and hCA II nitrate by Mangani's group.[71b] As seen from the data of Table 3, the spectra of the adducts of Co(II)-hCA II with some hydroxamates **7**−**45** (such as **8** and **19**) reported here are clearly of the pentacoordinated type mentioned above, with molar absorbances under 150 M^{-1} cm^{-1} and with an absorbance maxima distribution pattern as described above. The spectra of the adducts of the two hydroxamates are quite similar to those of the carboxylate adducts (acetate, benzoate) previously investigated.[70,72] On the contrary, the spectra of the adducts of Co(II)-hCA II with *N*-hydroxysulfonamides, such as **50** or **60** are typical for the tetrahedral geometry of the metal ion, proving that such derivatives bind similarly to the unsubstituted sulfonamides within the CA active site.[42,69a] The above data prompt us to propose the binding mode of hydroxamate inhibitors within the hCA II active site described schematically in Figure 4. Since the secondary sulfonamide moiety of such compounds would also possess good metal-coordinating properties,[55] we expect a bidentate binding, in which both the sulfonyl and hydroxamate moieties participate in the interaction with the metal ion (the inhibitor has been formulated as a monoanion, since at the pH at which the experiments have been performed (7.5−8.0) at least the hydroxamic acid group will be ionized).

In contrast to Co(II)-substituted CAs, metal substitution of MMPs or ChC has been much less investigated. Van Wart's group reported the preparation of Co(II)-ChC which possesses catalytic activity similar to that of the zinc enzyme,[69b] but no spectroscopic studies of E−I complexes were mentioned in this valuable study. Since ChC could not be crystallized for the moment, and its tridimensional structure is thus not available, electronic spectroscopic studies of Co(II)-substituted ChC might offer interesting information regarding the binding of inhibitors within the active site of this bacterial protease. Here we report for the first time the electronic spectroscopic studies of ChC and of its adducts with hydroxamate and *N*-hydroxysulfonamide inhibitors (Table 3). Co(II)-ChC possesses a pH-dependent electronic absorption spectrum, with a maximum centered at 585 nm and a shoulder at 530 nm, this spectrum being relatively similar to that of Co(II)-substituted carboxypeptidase A or thermolysin,[73−75] two enzymes in which the Zn(II) ion is coordinated − such as in ChC − by two histidines and a glutamate.[76] In the presence of hydroxamate (such as **8**, **11**, **23,** and **35**) or *N*-hydroxysulfonamide (such as **65** and **69**) inhibitors, major changes of this spectrum were evidenced: three absorption maxima instead the two mentioned above appeared: at 501−505, 562−563, and 597−598 nm, respectively. These spectra are of low intensity (molar absorbances around 80−120 M^{-1} cm^{-1} for the first two maxima and around 11−15 M^{-1} cm^{-1} for the last one), being characteristic of Co(II) in pentacoordinated geometry.[70−75] With the available data presented above, one can only state that similarly to the sulfonylated amino acid hydroxamates, *N*-hydroxysulfonamides also bind to the metal ion within the ChC active site and presumably also within the MMP active site, but the way in which they coordinate the metal ion is unknown for the moment.

Conclusions

We report a new class of effective CAIs: the sulfonylated amino acyl hydroxamates. The SARs for this class of CAIs are quite different from those of the structurally related sulfonylated hydroxamates with MMP inhibitory action. Best CA inhibitory activity was seen for compounds unsubstituted at the RSO_2NH-amino acyl moiety (with affinities in the low nanomolar range for isozymes CA I, CA II, and CA IV), with the less bulky Gly and Ala derivatives more active than the corresponding Val and Leu compounds. The perfluorophenylsulfonyl derivatives were the best inhibitors, followed by the *n*-perfluorobutylsulfonyl derivatives, which in turn were more active than the 4-methoxyphenylsulfonyl derivatives. Substitution at the secondary sulfonamide moiety with benzyl or substituted-benzyl moieties led to decreased CA inhibitory properties but to a drastic increase of the MMP inhibitory effects for the corresponding derivatives (against MMP-1, MMP-2, MMP-8, and MMP-9). The same is true regarding ChC inhibition with these compounds. We proposed a bidentate binding mode of these hydroxamates to the metal ion of the CA active site, similarly to that of CF_3-CONHOH reported previously. We also report here a novel zinc-binding function (SO_2NHOH) that may be incorporated into the molecule of novel types of MMPIs and ChC inhibitors. Although these derivatives were not optimized, some of them bind with K_i's of around 50 nM against the different investigated metalloproteases (MMPs/ChC). Electronic spectroscopic studies of the Co(II)-substituted CA and ChC showed undoubtedly that these new classes of inhibitors bind to the metal ion within the enzyme active site. Thus, we proved here that it is possible to design dual enzyme inhibitors, which interact both with the diverse MMPs and with the CAs.

Experimental Section

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). Analytical and preparative HPLC was performed on a reversed-phase C_{18} Bondapack column, with a Beckman EM-1760 instrument. Amino acids, sulfonyl halides, benzyl halides, EDCI, diisopropylcarbodiimide, Boc-ON, hydroxylamine, TFA, triethylamine, 5,5′-dithiobis(2-nitrobenzoic acid), FALGPA, buffers, and other reagents used in the syntheses were commercially available

compounds from Sigma-Aldrich or Acros (Milan, Italy). The thioester MMP substrate, AcProLeuGly-S-LeuLeuGlyOEt, was from Bachem. Acetonitrile, acetone, methylene chloride (from E. Merck, Florence, Italy), or other solvents used in the synthesis were doubly distilled and kept on molecular sieves in order to maintain them in anhydrous conditions.

General Procedure for the Preparation of Compounds 83. An amount of 10 mmol of sulfonyl halide (chlorides for the pentafluorophenyl and 4-methoxyphenylsulfonyl derivatives and fluoride for the pentafluorobutyl derivatives) and the stoichiometric amount of amino acid (Gly, L-Ala, l-Val or L-Leu) were suspended in 100 mL of acetone and magnetically stirred at 0−4 °C for 5 min. A solution obtained by dissolving 200 mg K_2CO_3 (20 mmol) in 15 mL of water was then added to the reaction mixture, and stirring was continued at room temperature for 4−6 h (TLC control). pH was brought to 7 with 5% aqueous HCl, the acetone was evaporated in vacuo, the obtained syrup was either directly recrystallized from ethanol−water (3/1−5/1, v/v) or poured into 50 mL of cold water and the obtained precipitate was filtered, dried and recrystallized as above. Yields were in the range of 80−95%.

General Procedure for the Preparation of Compounds 84. An amount of 5 mmol of sulfonyl amino acid **83**, 105 μL (7.5 mmol) of triethylamine and 135 mg (5.5 mmol) of Boc-ON were suspended in 20 mL of acetonitrile−water (1:1, v/v). The mixture became homogeneous in about 1 h, and stirring was continued at room temperature for additional 2−3 h (TLC control). The organic solvent was evaporated under reduced pressure, the residue was retaken in 25 mL of water, and the 2-hydroxyimino-2-phenylacetonitrile formed in the reaction was extracted with 2 × 10 mL of ethyl acetate. The aqueous layer was separated and acidified with a 5% citric acid solution till pH 4, and the Boc-protected compounds **84** were extracted in 100−150 mL of ethyl acetate. After evaporation of the organic solvent, they were recrystallized from ethanol. Yields of the protection step were in the range of 69−83%.

General Procedure for the Preparation of Compounds 85 and 86. An amount of 3 mmol of Boc-protected sulfonylated amino acid **84** was dissolved in 50 mL of anhydrous acetonitrile and magnetically stirred for 10 min at 0 °C. An amount of 570 mg (3 mmol) of EDCI·HCl was then added and the reaction mixture was magnetically stirred at room temperature for 15 min, then 90 μL (6 mM) of triethylamine and 210 mg of hydroxylamine·HCl (3 mmol) dissolved in 10 mL of water were added to the reaction mixture and stirring was continued for 8−10 h at 0−4 °C (TLC control). The solvent was evaporated in vacuo and the residue consisting of crude **85** was taken in 50 mL of methylene chloride and treated with 2 mL of TFA (magnetic stirring at room temperature for 30 min). The solvent and excess TFA were evaporated in vacuo; the residue was taken up in ethyl acetate (50 mL), dried over sodium sulfate, filtered, and purified by means of preparative HPLC (C_{18} reversed-phase μ-Bondapack or Dynamax-60A (25 × 250 mm) column; 80% acetonitrile/8% methanol/12% water, 30 mL/min). Yields were in the range of 45−65%.

General Procedure for the Preparation of *N*-Hydroxysulfonamides. The *N*-hydroxysulfonamides of type **46**−**80** were prepared as previously described by this group,[42] by reaction of arylsulfonyl halides or sulfonic acid anhydrides, with hydroxylamine in alcoholic-aqueous medium. An amount of 10 mmol of sulfonyl halide (chloride or fluoride) was dissolved/suspended in 50 mL of methanol and the stoichiometric amount (10 mmol, 0.69 g) of hydroxylamine hydrochloride dissolved in 10 mL of water was added. The mixture was magnetically stirred at 4 °C for 10 min, then the calculated amount of solid $NaHCO_3$ was added and stirring was continued for 4−6 h (TLC control) at room temperature, till all the sulfonyl halide was consumed in the reaction with the nucleophile. The solvent was evaporated under reduced pressure and the precipitated *N*-hydroxysulfonamides were recrystallized from ethanol or ethanol water. Yields were generally high (75−85%).

All the new compounds reported here were extensively characterized by means of standard chemical and physical methods (elemental analysis, within ±0.4% of the theoretical values; IR; ^{1}H and ^{13}C NMR spectroscopy) that confirmed their structure. Data for a representative compound of each series are shown below.

***N*-Pentafluorophenylsulfonyl-glycine, 83 (R1 = H, R = pentafluorophenyl):** white crystals, mp 178−9 °C; ^{1}H NMR (DMSO-d_6) δ 3.62 (s, 2H, CH_2 of Gly), 9.13 (br s, 1H, SO_2NH), 11.75 (br s, 1H, COOH); ^{13}C NMR (DMSO-d_6) δ 40.6 (s, CH$_2$ of Gly), 130.6 (s, *C*-3 of C_6F_5), 135.8 (s, *C*-2 of C_6F_5), 145.5 (s, *C*-4 of C_6F_5), 152.3 (s, *C*-1 of C_6F_5), 178.5 (s, CO$_2$H). Anal. Found: C, 31.62; H, 1.54; N, 4.33. $C_8H_4F_5NO_4S$ requires: C, 31.49; H, 1.32; N, 4.59.

***N*-Pentafluorophenylsulfonyl-glycine hydroxamate, 8**: white crystals, mp 211−2 °C; ^{1}H NMR (DMSO-d_6) δ 3.70 (s, 2H, CH_2 of Gly), 8.70 (br s, 1H, NHOH), 9.10 (br s, 1H, SO_2NH), 10.55 (br s, 1H, NHOH); ^{13}C NMR (DMSO-d_6) δ 40.4 (s, CH$_2$ of Gly), 130.5 (s, *C*-3 of C_6F_5), 135.3 (s, *C*-2 of C_6F_5), 145.8 (s, *C*-4 of C_6F_5), 152.2 (s, *C*-1 of C_6F_5), 176.8 (s, *C*ONHOH). Anal. Found: C, 30.24; H, 1.60; N, 8.63. $C_8H_5F_5N_2O_4S$ requires: C, 30.01; H, 1.57; N, 8.75.

***N*-*n*-Perfluorobutylsulfonyl-alanine hydroxamate, 13**: white crystals, mp 143−4 °C; ^{1}H NMR (DMSO-d_6) δ 1.51 (d, $^3J_{HH}$ = 6.1 Hz, 3H, CHCH_3 of Ala), 3.90 (q, 1H, CH of Ala), 8.65 (br s, 1H, NHOH), 9.18 (br s, 1H, SO_2NH), 10.50 (br s, 1H, NHOH); ^{13}C NMR (DMSO-d_6) δ 21.8 (s, CHCH$_3$ of Ala), 35.8 (s, CHCH$_3$ of Ala), 176.0 (s, *C*ONHOH). The four pentafluorobutyl carbons were not seen in the conditions of the experiments, similarly to the case of some perfluorobutyl-containing aromatic sulfonamides previously reported by Whiteside's group.[68] Anal. Found: C, 21.56; H, 1.75; N, 7.12. $C_7H_7F_9N_2O_4S$ requires: C, 21.77; H, 1.83; N, 7.25.

***N*-4-Methoxyphenylsulfonyl-*N*-benzyl-L-valine hydroxamate, 24**: white crystals, mp 222−4 °C; ^{1}H NMR (DMSO-d_6) δ 1.12 (d, $^3J_{HH}$ = 6.7 Hz, 6H, CH(CH_3)$_2$ of Val), 2.23−2.55 (m, 1H, CH(CH$_3$)$_2$ of Val), 3.45 (s, 3H, CH_3OC$_6$H$_4$), 3.77 (d, $^3J_{HH}$ = 4.4 Hz, 1H, NCHCO of Val), 3.82 (s, 2H, CH_2 of benzyl), 7.13−7.60 (m, 7H, H_{ortho} of CH$_3$OC$_6$H$_4$ and H_{arom} of Ph), 8.00 (d, $^3J_{HH}$ = 8.1 Hz, 2H, H_{meta} of CH$_3$OC$_6$H$_4$), 8.71 (br s, 1H, NHOH), 10.51 (br s, 1H, NHOH); ^{13}C NMR (DMSO-d_6) δ 17.3 (s, CH(CH$_3$)$_2$ of Val), 30.1 (s, CH(CH$_3$)$_2$ of Val), 33.4 (s, CH$_3$-OC$_6$H$_4$), 41.4 (s, CH$_2$ of benzyl), 61.1 (s, NHCH of Val), 130.3 (s, C_{meta} of CH$_3$OC$_6$H$_4$), 131.8 (s, C_{para} of Ph), 133.5 (s, C_{meta} of Ph), 134.78 (s, C_{ortho} of Ph), 135.5 (s, C_{ortho} of CH$_3$OC$_6$H$_4$), 141.6 (s, C_{ipso} of Ph), 145.0 (s, C_{ipso} of CH$_3$OC$_6$H$_4$), 146.3 (s, C_{para} of CH$_3$OC$_6$H$_4$), 174.2 (s, *C*ONHOH). Anal. Found: C, 47.81; H, 6.19; N, 9.08. $C_{12}H_{18}N_2O_5S$ requires: C, 47.67; H, 6.00; N, 9.27.

***N*-Pentafluorophenylsulfonyl-*N*-4-nitrobenzyl-glycine hydroxamate, 35**: white crystals, mp 165−7 °C; ^{1}H NMR (DMSO-d_6) δ 3.71 (s, 2H, CH_2 of Gly), 4.39 (s, 2H, CH_2 of benzyl), 7.21−7.67 (m, 2, H_{ortho} of O_2NC$_6$H$_4$), 8.21 (d, 2H, H_{meta} of O_2NC$_6$H$_4$), 8.70 (br s, 1H, NHOH), 10.58 (br s, 1H, NHOH); ^{13}C NMR (DMSO-d_6) δ 40.6 (s, CH$_2$ of Gly), 45.6 (s, CH$_2$ of benzyl), 123.8 (s, C_{meta} of O_2NC$_6$H$_4$), 129.4 (C_{ortho} of O_2-NC$_6$H$_4$), 130.7 (s, C_{meta} of C_6F_5), 135.9 (s, C_{ortho} of C_6F_5), 144.3 (s, C_{ipso} of O_2NC$_6$H$_4$), 145.5 (s, C_{ipso} of C_6F_5), 147.9 (s, C_{para} of O_2NC$_6$H$_4$), 152.3 (s, C_{para} of C_6F_5), 178.3 (s, *C*ONHOH). Anal. Found: C, 39.77; H, 2.39; N, 9.02. $C_{15}H_{10}F_5N_3O_6S$ requires: C, 39.57; H, 2.21; N, 9.23.

***N*-4-Methoxyphenylsulfonyl-*N*-2-nitrobenzyl-L-alanine hydroxamate, 39**: pale yellow crystals, mp 194−5 °C; ^{1}H NMR (DMSO-d_6) δ 1.56 (d, $^3J_{HH}$ = 6.5 Hz, 3H, CHCH_3 of Ala), 3.45 (s, 3H, CH_3OC$_6$H$_4$), 3.81 (s, 2H, CH_2 of benzyl), 3.98 (q, 1H, CH of Ala), 7.14−7.68 (m, 6H, H_{ortho} of CH$_3$OC$_6$H$_4$ and H_{arom} of 2-O_2N-C$_6$$H_4$), 8.07 (d, $^3J_{HH}$ = 8.1 Hz, 2H, H_{meta} of CH$_3$-OC$_6$H$_4$), 8.78 (br s, 1H, NHOH), 10.56 (br s, 1H, NHOH); ^{13}C NMR (DMSO-d_6) δ 20.5 (s, CHCH$_3$ of Ala), 33.1 (s, CH$_3$OC$_6$H$_4$), 34.9 (s, CHCH$_3$ of Ala), 44.4 (s, CH$_2$ of benzyl), 127.0 (s, C-5 of 2-O_2N-C$_6$H$_4$), 129.3 (C-4 of 2-O_2N-C$_6$H$_4$), 129.9 (C-3 of 2-O_2N-C$_6$H$_4$), 130.4 (s, C_{meta} of CH$_3$OC$_6$H$_4$), 131.3 (C-6 of 2-O_2N-C$_6$H$_4$), 134.5 (C-2 of 2-O_2N-C$_6$H$_4$), 135.3 (C-1 of 2-O_2N-C$_6$H$_4$), 135.7 (s, C_{ortho} of CH$_3$OC$_6$H$_4$), 146.8 (s, C_{ipso} of CH$_3$OC$_6$H$_4$), 148.4 (s, C_{para} of CH$_3$OC$_6$H$_4$), 175.3 (s, *C*ONHOH). Anal. Found: C, 50.03; H, 4.80; N, 10.11. $C_{17}H_{19}N_3O_7S$ requires: C, 49.87; H, 4.68; N, 10.26.

CA Preparations and Assay. 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 et al.[61] (the two plasmids were a gift from Prof. Sven Lindskog, Umea University, Sweden). Cell growth conditions were those described by this group,[62] and enzymes were purified by affinity chromatography according to the method of Khalifah et al.[63] 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 29.30 kDa for CA II, respectively.[64,65] CA IV was isolated from bovine lung microsomes as described by Maren et al., and its concentration has been determined by titration with ethoxzolamide.[66]

Initial rates of 4-nitrophenyl acetate hydrolysis catalyzed by different CA isozymes were monitored spectrophotometrically, at 400 nm, with a Cary 3 instrument interfaced with an IBM-compatible PC.[45] Solutions of substrate were prepared in anhydrous acetonitrile; the substrate concentrations varied between 2×10^{-2} and 1×10^{-6} M, working at 25 °C. A molar absorption coefficient ϵ of 18 400 M^{-1} cm^{-1} was used for the 4-nitrophenolate formed by hydrolysis, in the conditions of the experiments (pH 7.40), as reported in the literature.[45] Nonenzymatic hydrolysis rates were always subtracted from the observed rates. Duplicate experiments were done for each inhibitor concentration, and the values reported throughout the paper are the mean of such results. Stock solutions of inhibitor (1 mM) were prepared in distilled−deionized water with 10−20% (v/v) DMSO (which is not inhibitory at these concentrations) 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, to allow for the formation of the E−I complex. The inhibition constant K_i was determined as described by Pocker and Stone.[45] Enzyme concentrations were 3.6 nM for hCA II, 8.8 nM for hCA I, and 27 nM for bCA IV (this isozyme has a decreased esterase activity[14] and higher concentrations had to be used for the measurements).

MMP and ChC Preparations. Human purified MMPs (MMP-1, MMP-2, MMP-8 and MMP-9) were purchased from Calbiochem (Inalco, Milano, Italy). They were activated[67] in the assay buffer by adding bovine trypsin (from Sigma, 50 μL, 0.6 mg/mL) to the proenzyme, followed by incubation at 37 °C for 10 min. The trypsin was then inactivated with aprotinin (50 μL, 1.2 mg/mL). Initial rates for the hydrolysis of the thioester substrate AcProLeuGly-S-LeuLeuGlyOEt, coupled to the reaction with 5,5′-dithiobis(2-nitrobenzoic acid) were used for assessing the catalytic activity and inhibition of the four MMPs mentioned above, by the spectrophotometric method of Powers and Kam,[46a] and Johnson et al.[46b] The change of absorbance (ϵ = 19 800 M^{-1} cm^{-1})[46a] at 405 nm was monitored continuously at room temperature, using a Cary 3 spectrophotometer interfaced with a PC. A typical 100-μL reaction contained 50 mM MES, pH 6.0, 10 mM $CaCl_2$, 100 μM substrate, 1 mM 5,5′-dithiobis(2-nitrobenzoic acid) and 5 nM MMP. For the K_i determinations, DMSO solutions of the inhibitor were included in the assay, resulting in a final concentration of 2% DMSO in the reaction mixture. In these conditions, K_i values varied from 5−10% in replicate experiments. K_i's were then determined by using Easson−Stedman[56] plots and a linear regression program.

Clostridium histolyticum highly purified collagenase and its substrate FALGPA (furanacryloyl-leucyl-glycyl-prolyl-alanine) were purchased from Sigma Chemical Co. (Milano, Italy), and their concentrations were determined from the absorbance at 280 nm and the extinction coefficients furnished by the supplier. The activity of such preparations was in the range of 10 NIH units/mg solid. The potency of standard and newly obtained inhibitors was determined from the inhibition of the enzymatic (amidolytic) activity of the collagenase, at 25 °C, using FALGPA as substrate, by the method of van Wart and Steinbrink.[47] The substrate was reconstituted as 5 mM stock in 50 mM Tricine buffer, 0.4 M NaCl, 10 mM $CaCl_2$, pH 7.50. The rate of hydrolysis was determined from the change in absorbance at 324 nm using an extinction coefficient for FALGPA ϵ_{305} = 24 700 M^{-1} cm^{-1} in the above-mentioned reaction buffer.[47] Measurements were made using a Cary 3 spectrophotometer interfaced with a PC. Initial velocities were thus estimated using the direct linear plot-based procedure reported by van Wart and Steinbrink.[47] K_i's were then determined according to Easson−Stedman[56] plots and a linear regression program.

Spectroscopic Studies on Co(II)-Substituted Enzymes. Co(II)-hCA II was prepared as described in ref 69a, whereas Co(II)-ChC by a modification of the method described in ref 69b by removing zinc from the native enzyme in the presence of 50 mM pyridine-2,6-dicarboxylic acid, followed by dialysis against metal-free 50 mM Tris-H_2SO_4 buffer and addition of the stoichiometric amount of Co(II) chloride. Electronic spectra were registered with a Cary 3 spectrophotometer, at 25 °C (in the buffer mentioned above), in the range of 400−800 nm, working at enzyme concentrations of 0.1−0.4 mM and the pH values specified in each case (generally of 6.5−8 pH units). The E−I adducts of the two cobalt enzymes mentioned above were obtained by spectral titration of the pure enzyme with inhibitors, until reaching an inhibitor concentration of 0.1−2 mM in the spectral cuvette. The absorbance values of these spectra were converted to extinction coefficients after background subtraction and normalization based on absorbance averages at 550−575 nm for Co(II)-hCA II and 560−585 nm for Co(II)-ChC, respectively.

Acknowledgment. This research was financed by EU Grant ERB CIPDCT 940051. Special thanks are addressed to Drs. Giovanna Mincione, Angela Casini, and Marc A. Ilies for their assistance in different phases of this project.

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(1) This paper is part 93 of the series. Preceding part: Scozzafava, A.; Supuran, C. T. Carbonic anhydrase inhibitors. Synthesis of N-Morpholyl-thiocarbonylsulfenylamino aromatic/ heterocyclic sulfonamides and their interaction with isozymes I, II and IV. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 1117−1120.

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JM000027T