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Protease Inhibitors. Part 8: Synthesis of Potent *Clostridium histolyticum* Collagenase Inhibitors Incorporating Sulfonylated L-Alanine Hydroxamate Moieties

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Abstract—A series of hydroxamates was prepared by reaction of alkyl/arylsulfonyl halides with *N*-2-chlorobenzyl-L-alanine, followed by conversion of the COOH moiety to the CONHOH group, with hydroxylamine in the presence of carbodiimides. Other structurally related compounds were obtained by reaction of *N*-2-chlorobenzyl-L-alanine with aryl isocyanates, arylsulfonyl isocyanates or benzoyl isothiocyanate, followed by the similar conversion of the COOH into the CONHOH moiety. The new compounds were assayed as inhibitors of the *Clostridium histolyticum* collagenase, ChC (EC 3.4.24.3), a bacterial zinc metallo-peptidase which degrades triple helical collagen as well as a large number of synthetic peptides. The prepared hydroxamate derivatives proved to be 100–500 times more active collagenase inhibitors than the corresponding carboxylates. Substitution patterns leading to best ChC inhibitors (both for carboxylates as well as for the hydroxamates) were those involving perfluoroalkylsulfonyl- and substituted-arylsulfonyl moieties, such as pentafluorophenylsulfonyl; 3- and 4-protected-aminophenylsulfonyl-; 3- and 4-carboxyphenylsulfonyl-; 3-trifluoromethyl-phenylsulfonyl; as well as 1- and 2-naphthyl-, quinoline-8-yl- or substituted-arylsulfonylamidocarboxyl moieties among others. Similarly to the matrix metalloproteinase (MMP) hydroxamate inhibitors, ChC inhibitors of the type reported here must incorporate hydrophobic moieties at the $P_{2'}$ and $P_{3'}$ sites, in order to achieve tight binding to the enzyme. This study also proves that the 2-chlorobenzyl moiety, investigated here for the first time, is an efficient $P_{2'}$ anchoring moiety for obtaining potent ChC inhibitors.

Introduction

During our studies in the design and synthesis of anti-glaucoma agents with topical action,[1–3] it was frequently observed that experimental animals used in the work (albino rabbits) developed eye infections due to bacterial pathogens, among which *Clostridium histolyticum* was the prevalent species. A literature survey proved that bacterial corneal keratitis,[4] a condition leading to serious complications for which efficient cures are difficult to envisage at the moment,[4] has indeed been reported to be associated with a highly increased collagenase activity in the ocular tissues, in human patients and experimental animals.[5] Thus, it appeared of interest to investigate in detail the design of bacterial collagenase inhibitors with putative ophthalmologic applications.

As amino acid and oligopeptide hydroxamates were reported to act as powerful inhibitors for a large number of metallo-enzymes important as targets in the drug design (such as the matrix metalloproteinases, MMP's;[6–9] thermolysin and ellastase;[10] leucine aminopeptidase;[11] carboxypeptidase A;[12] leukotriene A4 hydrolase;[13] angiotensin I-converting enzyme;[14] neurotensin-degrading enzymes;[15] endothelin-converting enzyme;[16] or UDP-3-O-(R-3-hydroxymyristoyl)-*N*-acetylglucosamine deacetylase[17–19] among others), we decided to investigate such derivatives as anti-bacterial corneal keratitis agents. Some of the above-mentioned derivatives were also shown to possess strong anti-bacterial activity since by inhibiting some of the above mentioned enzymes, they interfere with lipid A biosynthesis and inhibit the growth of Gram-negative bacteria.[17–19] Other compounds of this type were also reported to act as anti-HIV agents in vitro.[20] But the most important applications in drug design of the amino acid/oligopeptide hydroxamates is related to their use for the development of MMP inhibitors as anti-cancer[21–25] or anti-arthritis[7,26–28] drugs. The 23

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MMP's presently known[7,29] are involved in tissue remodeling connected with tumor invasion and joint destruction.[6,7,22–26] Synthetic high affinity inhibitors for some of these enzymes, such as the four vertebrate collagenases (MMP's 1, 8, 13 and 18), stromelysins 1 and 2 (MMP's 3 and 10, respectively) or the gelatinases A and B (MMP's 2 and 9) were much investigated in the last period, in order to develop novel pharmacological agents of the hydroxamate type.[6,7,25–29] Not the same situation is true for the inhibitors of bacterial collagenases, such as for instance, the enzyme isolated from *C. histolyticum*,[30–32] which was much less investigated. This collagenase (EC 3.4.24.3) is a 116 kDa protein belonging to the M31 metalloproteinase family,[29] being able to hydrolyze triple helical regions of collagen under physiological conditions, as well as an entire range of synthetic peptide substrates.[30–32] In fact the crude homogenate of *Clostridium histolyticum*, which contains several distinct collagenase isozymes, is the most efficient system known for the degradation of connective tissue,[31,32] being also involved in the pathogenicity of this and related clostridia, such as *C. perfringens*, which causes human gas gangrene and food poisoning among others.[33]

Similar to the vertebrate MMP's,[7,29–35] *C. histolyticum* collagenase (abbreviated as ChC) has the conserved HExxH zinc-binding motif, which in this specific case is His[415]ExxH, with the two histidines (His 415 and His 419) acting as Zn(II) ligands; whereas the third ligand seems to be Glu 447,[30–35] (in the case of the vertebrate MMP's the zinc ion is coordinated by three histidines, His 218, His 222 and His 228), and a water molecule/hydroxide ion acting as a nucleophile in the hydrolytic scission.[5–9,30–35] Similar to the MMP's, ChC is also a multidomain protein, consisting of four segments, S1, S2a, S2b and S3,[35] with S1 incorporating the catalytic domain.

Thus, we hypothesized that amino acid hydroxamates and some of their derivatives, which strongly inhibit MMP's (collagenases, gelatinases, stromelysins, etc.), would also act as potent ChC inhibitors. We also hypothesized that the use of a collagen shield, soaked in an antibiotic agent specific for the collagen-degrading bacteria, would have a double benefit for the patients affected by bacterial corneal keratitis: (i) the collagenase inhibitor would kill (or impair the growth of) bacteria present on the cornea, improving and accelerating healing of the keratitis; (ii) the (protective) collagen shield would acquire an augmented stability, as its degradation by the secreted collagenases would be delayed, promoting/accelerating in this way the healing of the wound. On the other hand, the topical application of such a pharmacological agent would avoid serious systemic toxicity problems.

In this paper we report the preparation of a series of ChC inhibitors incorporating alkyl/arylsulfonamido-*N*-2-chlorobenzyl-L-alanine hydroxamate as well as arylsulfonylureido-/arylureido-*N*-2-chlorobenzyl-L-alanine hydroxamate moieties in their molecule. Some of the new compounds, assayed for the inhibition of purified ChC, showed high affinity for the enzyme (in the nanomolar range), behaving as very potent inhibitors. The results of the in vivo experiments as anti-corneal bacterial keratitis agents with some of the compounds synthesized in the present paper will be reported elsewhere.

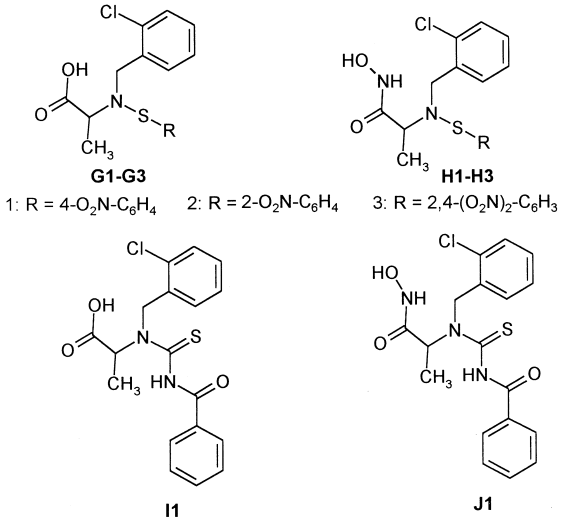
Results

Synthesis

The key intermediate **3** for the synthesis of new collagenase inhibitors reported here, was prepared by reaction of 2-chlorobenzyl-chloride (**1**) with L-alanine (**2**). Compounds **A1**–**A34** were then obtained by the reaction of **3** with alkyl/arylsulfonyl halides **4**, as shown in Scheme 1 and Table 1. Conversion of the carboxylic acids **A1**–**A34** into the corresponding hydroxamates **B1**–**B34** was done with hydroxylamine and diisopropyl carbodiimide (Scheme 1).

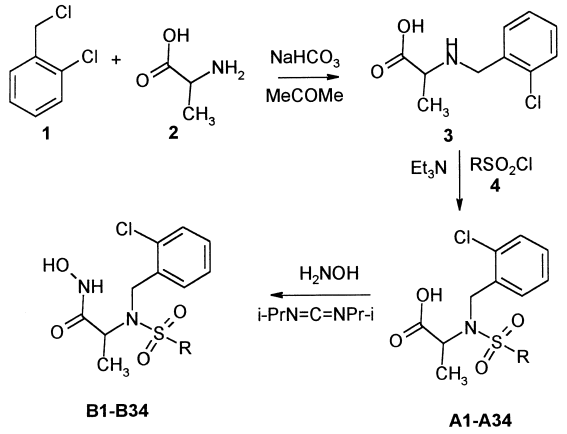
Another series of derivatives (**C1**–**C4**; **D1**–**D4** and **E1**–**E6**; **F1**–**F6**) was obtained by reaction of arylsulfonylisocyanates (**5**) or aryl isocyanates (**6**) with *N*-2-chlorobenzyl-glycine (**3**), followed by the conversion of the COOH moiety into the CONHOH one, as described above (Schemes 2 and 3).

By applying synthetic strategies related to the previously described ones, the sulfenamido derivatives **G1**–**G3** and **H1**–**H3**, as well as the thioureas **I1** and **J1** were also obtained.

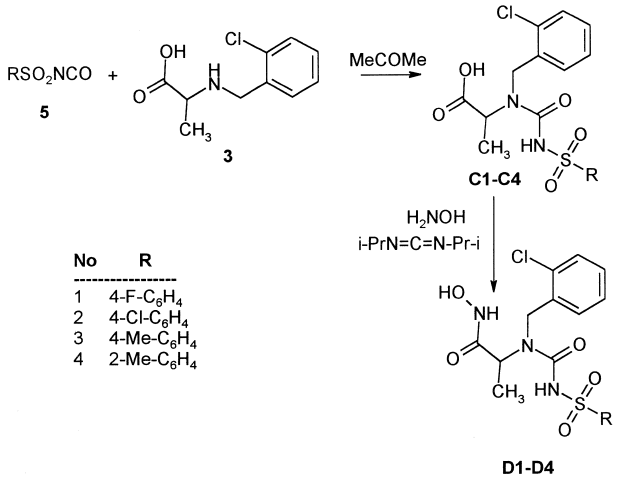


Collagenase inhibition

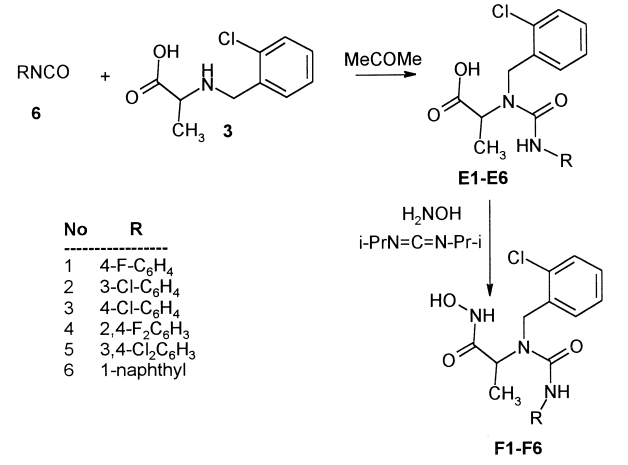
Inhibition data against highly purified ChC type VII, with the newly prepared carboxylic and hydroxamic acids are shown in Tables 1 and 2. The chromogenic substrate FALGPA (2-furanacryloyl-L-Leu-Gly-L-Pro-L-Ala) was used in the assay, with the spectrophotometric method of Van Wart and Steinbrink.[30] Inhibition data with some standard ChC inhibitors



Scheme 1.



Scheme 2.



Scheme 3.

Table 1. Inhibition of ChC with the carboxylic acids **A1**–**A34** and the corresponding hydroxamates **B1**–**B34**

R	Compound	K_I[a] (μM)	Compound	K_I[a] (nM)
CH_3	**A1**	21	**B1**	92
CF_3	**A2**	3.3	**B2**	76
CCl_3	**A3**	5.2	**B3**	71
n-C_4F_9-	**A4**	3.0	**B4**	11
n-C_8F_{17}	**A5**	1.5	**B5**	8
Me_2N-	**A6**	40	**B6**	77
C_6H_5-	**A7**	24	**B7**	58
$PhCH_2$-	**A8**	16	**B8**	54
4-F-C_6H_4-	**A9**	12	**B9**	36
4-Cl-C_6H_4-	**A10**	13	**B10**	35
4-Br-C_6H_4-	**A11**	10	**B11**	33
4-I-C_6H_4-	**A12**	10	**B12**	30
4-CH_3-C_6H_4	**A13**	18	**B13**	45
4-O_2N-C_6H_4-	**A14**	5.1	**B14**	15
3-O_2N-C_6H_4-	**A15**	5.0	**B15**	11
2-O_2N-C_6H_4-	**A16**	4.9	**B16**	16
3-Cl-4-O_2N-C_6H_3-	**A17**	3.1	**B17**	10
4-AcNH-C_6H_4-	**A18**	3.0	**B18**	11
4-BocNH-C_6H_4-	**A19**	2.7	**B19**	9
3-BocNH-C_6H_4-	**A20**	2.4	**B20**	8
C_6F_5-	**A21**	0.4	**B21**	5
3-CF_3-C_6H_4	**A22**	0.5	**B22**	6
2,5-$Cl_2C_6H_3$	**A23**	4.1	**B23**	15
4-CH_3O-C_6H_4-	**A24**	6.0	**B24**	22
2,4,6-$(CH_3)_3$-C_6H_2-	**A25**	6.8	**B25**	17
4-CH_3O-3-BocNH-C_6H_3-	**A26**	3.0	**B26**	8
2-HO-3,5-Cl_2-C_6H_2-	**A27**	2.4	**B27**	9
3-HOOC-C_6H_4-	**A28**	4.1	**B28**[b]	8
4-HOOC-C_6H_4-	**A29**	3.8	**B29**[b]	7
1-Naphthyl	**A30**	1.6	**B30**	8
2-Naphthyl	**A31**	1.5	**B31**	9
5-Me_2N-1-naphthyl-	**A32**	2.1	**B32**	10
2-Thienyl	**A33**	2.4	**B33**	11
Quinoline-8-yl	**A34**	2.0	**B34**	7

[a]K_I-s values were obtained from Dixon plots using a linear regression program, from at least three different assays.
[b]The C_6H_4-COOH moiety transformed into C_6H_4-CONHOH.

reported in the literature are also provided for comparison in Table 3.

Discussion

A literature search showed that a relatively small number of bacterial collagenase inhibitors have been reported up to now.[24,36–42] Since the ChC enzyme catalyzes the cleavage of the Xaa-Gly peptide bond of the repeating sequence of collagen: -Gly-Pro-Xaa-Gly-Pro-Xaa- (Xaa = amino acid residue), it appears that the S_3, S_2 and S_1 subsites of the enzyme are occupied by Gly, Pro and Xaa, respectively.[30–36] Analogously, the $S_{1'}$, $S_{2'}$ and $S_{3'}$ subsites are also occupied by Gly, Pro and Xaa, respectively.[30–36] Thus, many of the reported inhibitors of ChC are aldehyde or ketone-type substrate analogues, such as Pro_6-Gly-L-Pro-GlyH (GlyH = glycine aldehyde), with a K_I of 340 μM;[36] phosphoric and phosphonic amides, such as *iso*-amylphosphonyl-Gly-L-Pro-L-Ala (K_I of 16 μM);[37] thiols such as HS-CH_2CH_2CO-Pro-Xaa (K_I of the best compounds around 0.2 μM);[38–40] phosphonamide peptides of the type *p*-nitrophenethyl-PO(OH)-Gly-Pro-Xaa (K_I of the best compound, with Xaa = 2-aminohexanoic acid was of 5 nM).[41,42] As shown from the above data, either the obtained inhibitors are relatively weak, or the high affinity ones are phosphorus based ligands which are not suitable for the development of pharmaceutical agents, due to their high toxicity.

Table 2. Inhibition of ChC with the carboxylic acids of types **C, E, G, I** and the corresponding hydroxamates of types **D, F, H, J**

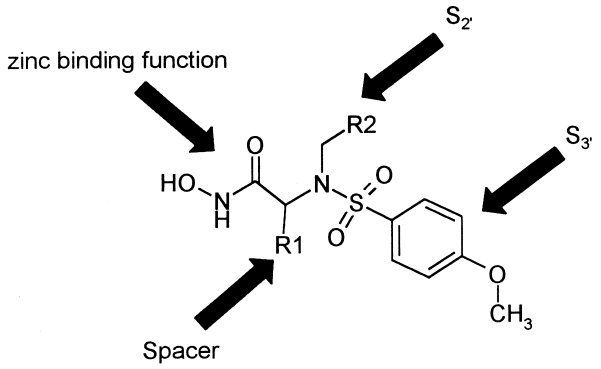
R	Compound	K_I[a] (μM)	Compound	K_I[a] (nM)
4-F-C_6H_4-	**C1**	3.6	**D1**	9
4-Cl-C_6H_4-	**C2**	3.4	**D2**	8
4-CH_3-C_6H_4-	**C3**	4.1	**D3**	11
2-CH_3-C_6H_4-	**C4**	4.0	**D4**	10
4-F-C_6H_4-	E1	7.4	**F1**	18
3-Cl-C_6H_4-	**E2**	7.6	**F2**	17
4-Cl-C_6H_4-	**E3**	6.8	**F3**	15
2,4-F_2-C_6H_3-	**E4**	5.5	**F4**	14
3,4-$Cl_2C_6H_3$	**E5**	5.8	**F5**	15
1-Naphthyl	**E6**	5.0	**F6**	11
4-O_2N-C_6H_4-	**G1**	6.0	**H1**	13
2-O_2N-C_6H_4-	**G2**	6.1	**H2**	12
2,4-(O_2N)$_2$-C_6H_3-	**G3**	6.1	**H3**	11
—	**I1**	0.9	**J1**	6

[a]K_I-s values were obtained from Dixon plots using a linear regression program, from at least three different assays.

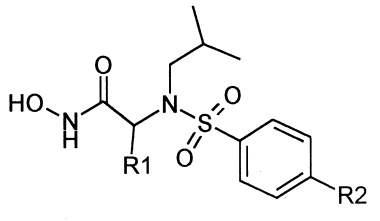
Table 3. Inhibition of ChC with standard inhibitors

Compound	K_I[a] (μM)
Pro_6-Gly-Pro-GlyH	340
i-C_5H_{11}PO(OH)GlyProAla	16
p-$O_2NC_6H_4CH_2CH_2$PO(OH)GlyPro-2AX[b]	0.005

[a]K_I-s values were obtained from Dixon plots using a linear regression program, from at least three different assays.
[b]2AX = 2-aminohexanoic acid.



7a: R1 = H, Me, *i*-Pr; R2 = Ph, *i*-Pr



7b: R1 = $CH_2CH_2SCH_2Ph$; R2 = OMe

7c: R1 = $CH_2CH_2SCH_2Ph$; R2 = Ph

Thus, the lead molecule that we used for designing the novel ChC inhibitors reported here was not of the type mentioned above. Taking into account the strong MMP inhibitory properties of some arylsulfonyl–amino hydroxamic acids of type **7a**–**7c** recently reported by Parker's[26] and Hanessian's[21] groups, we decided to use such derivatives as lead molecules. In the first of the above mentioned study[26] it was observed that best inhibitors of type **7a** (against mouse macrophage metalloelastase) were those incorporating: (i) Gly, Ala or Val as spacers between the zinc-binding function (the hydroxamic acid moiety) and the $P_{2'}$ site; (ii) benzyl or isobutyl moieties at $S_{2'}$, and (iii) arylsulfonyl moieties at $S_{3'}$.[26] It should be noted that a relatively small number of arylsulfonyl moieties were investigated in the above-mentioned studies,[21,26] the greatest number of the synthesized inhibitors **7** containing the 4-methoxybenzenesulfonyl or biphenylsulfonyl moieties. The proposed interaction sites between an inhibitor of type **7a** and the active site of the enzyme is also shown schematically in the formula of the inhibitor **7a**.

Considering the above-mentioned findings,[21,26] we opted for the following structural elements in the design of the ChC inhibitors reported in the present study: (i) a strong zinc-binding function (of the carboxylic acid, or better, hydroxamic acid types);[6,7] (ii) a relatively compact spacer between this function and the rest of the molecule, i.e. L-Ala;[6,26] (iii) a variant of the already optimized[26] benzyl group at the $S_{2'}$ site, which has not been investigated previously, i.e. the 2-chlorobenzyl moiety. The presence of the halogen atom substituting the $S_{2'}$ moiety was thought to be important for binding of the inhibitor to the enzyme, due to its steric and especially electronic effects that would clearly influence other properties of the inhibitor, such as the pK_a value of the hydroxamic acid group; (iv) variable alkyl/arylsulfonyl-; arylsulfonyl-ureido/arylureido-; or arylsulfenyl, moieties at $S_{3'}$.

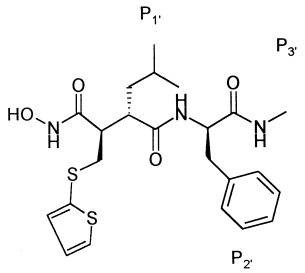
The new compounds reported here were obtained by non-exceptional synthetic procedures, as outlined in Schemes 1–3. These involved reaction of *N*-2-chlorobenzyl-L-alanine (**3**) with alkyl/arylsulfonyl chlorides,[43] arylisocyanates,[44] arylsulfonyl-isocyanates[45] or benzoyl-isothiocyanate, followed by conversion of the COOH moiety to the hydroxamate one.[46] Related synthetic strategies led to some sulfenamides of type **G, H**, as well as to the thioureas **I, J**.

ChC inhibition data with the new compounds reported here as well as other reported inhibitors are shown in Tables 1–3.

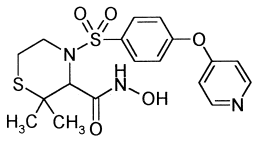
The following should be noted regarding ChC inhibition data of Tables 1–3 with the new compounds and standard inhibitors: (i) all hydroxamates were 100–500 times more active as ChC inhibitors as compared to the corresponding carboxylic acids, probably due to the enhanced Zn(II) coordinating properties of the CONHOH moiety (bidentate binding) as compared to the COOH group (generally monodentate binding to the zinc ion).[6–9] (ii) Potent inhibitors were obtained from all classes of hydroxamates investigated here, such as the alkyl/arylsulfonyl-*N*-2-chlorobenzyl-Ala derivatives (**B5**, **B20**–**B22**, **B26**, **B27**, **B29**, etc), the arylsulfonylureas-

and arylureas (such as **D2**, **F5**, **F6**), the sulfenamidobenzyl-Ala derivatives (such as **H2**, **H3**) or the thiourea **J1**. Thus, it seems that the $S_{3'}$-binding moiety of the arylsulfonamide type, previously investigated for the obtaining of MMP inhibitors of type **7**,[21,26] can be efficiently substituted by related moieties such as alkylsulfonyl-; arylsulfenyl-; arylsulfonylureido-; arylureido- or benzoyl-thioureido, without loss of the ChC inhibitory properties. (iii) In the subseries of alkyl/arylsulfonamido derivatives (of types **A,B(1—34)**) best ChC inhibitory properties were correlated with the presence of perfluoroalkylsulfonyl- (**B4**, **B5**), perfluorophenylsulfonyl- (**B21**), 3-trifluoromethylphenylsulfonyl- (**B22**); 3-chloro-4-nitro-phenylsulfonyl- (**B17**); 3- or 4-protected-aminophenylsulfonyl- (**B18**–**B20**; **B26**); 3- or 4-carboxy-phenylsulfonyl- (**B28**, **B29**), 1- or 2-naphthylsulfonyl as well as 8-quinolinesulfonyl moieties (**B30**–**B32**; **B34**). All these derivatives possessed inhibition constants in the range of 5–12 nM against ChC, being among the most potent such inhibitors ever reported. A second group of sulfonamide inhibitors, containing moieties such as 4-bromophenyl; 4-iodophenyl; 2-, 3- or 4-nitrophenyl; 2,5-dichlorophenyl-; 2,4,6-trimethylphenyl-; 4-methoxyphenyl- or 2-thienyl, substituting the *N*-benzyl-glycine hydroxamate, behaved as medium potency inhibitors, with affinities in the 15–30 nM range (Table 1). The least active sulfonamides were those containing methyl-; trihalomethyl-; dimethylamino-; phenyl- and benzyl moieties (Table 1). (iv) The arylsulfonylureido compounds **D1**–**D4** were more active than the corresponding arylsulfonyl derivatives (compare for instance **D1** with **B9**; **D2** and **B10**, etc), acting as strong ChC inhibitors. Similarly behaved were the ureas of type **F**, and the sulfenamides of type **H**, except for **F5** and **F6**, as well as **H2** and **H3**, which are strong inhibitors. A very potent inhibitor is the thiourea derivative **J1** (Table 2). By comparing the data of Tables 1–3, it is obvious that the compounds reported in the present study are among the best ChC inhibitors obtained up to now, since other such derivatives usually had affinities in the micromolar range (except for the phosphonic acid derivative mentioned in Table 3, which possessed an affinity of the same order of magnitude as that of our compounds).

Although ChC (or its catalytic domain) could not be crystallized up to now (in our or in other laboratories), and the precise binding of inhibitors cannot be inferred from X-ray crystallographic data, several important contributions in the field of the MMP's have been registered recently, and this might be useful in interpreting our inhibition data. Thus, Bode's, Moroder's and Tschesche's groups reported[8,9,47–49] several X-ray crystallographic studies for the interaction of some types of hydroxamate inhibitors with the catalytic domain of MMP-8, a collagenase from vertebrates, inhibited among others with a K_I of 10 nM by one of the collagenase inhibitors in clinical study, batimastat (**8**).[50,51] Another such derivative **9** (AG-3340), was recently reported by the Agouron group to possess broad antitumor and antiangiogenic activities in many tumor models as well as in patients with advanced malignancies of the lung and prostate.[52–54]



8: Batimastat



9: AG-3340

These studies[8,9,47–49] showed that batimastat is bidentately coordinated to the Zn(II) ion of the enzyme, through the hydroxamate OH moiety and hydroxamate CO group. The OH and NH of the hydroxamate moiety participate in supplementary interactions with the enzyme, forming hydrogen bonds with Glu 198 and Ala 161.[47] The hydrophobic residues in the $P_{1'}$ (isobutyl) and $P_{2'}$ (benzyl) positions are also critical for the formation of a strong E-I adduct: thus, the leucine side chain of $P_{1'}$ extends into the $S_{1'}$ pocket, making hydrophobic contacts with several amino acid residues such as His 197; Pro 217 and Val 194, whereas the phenyl ring of $P_{2'}$ interacts with the side chains of Ile 159, Val 129 and Pro 217. Although the class III collagenase inhibitors (in the classification of Babine and Bender[6]) of the sulfonamide type, to which the compounds reported here presumably belong, were much less investigated crystallographically, it is assumed that the general binding mode illustrated above is also valid for them, although some differences were also evidenced.[6,21,26] Based on these observations, we propose a similar binding mode of the sulfonamide inhibitors reported here to ChC, as showed schematically in Figure 1.

The inhibitor probably coordinates bidentately to the Zn(II) ion of ChC, whereas the hydrophobic moieties from the $P_{2'}$ and $P_{3'}$ sites (2-chlorobenzyl and pentafluorophenyl, respectively) participate in hydrophobic contacts which assure the strong affinity of this inhibitor for ChC (the inhibitor **B21** for which the schematic binding is shown above has a K_I of 5 nM).

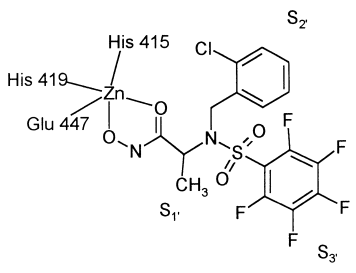


Figure 1. Proposed schematic binding of inhibitor **B21** within the active site of ChC.

Conclusion

We describe here a novel class of strong inhibitors of the zinc protease EC 3.4.24.3, a collagenase from *C. histolyticum*. As no X-ray crystallographic structure of this enzyme is available, the drug design has been realized by utilizing X-ray data for the MMP's, related enzymes which degrade the extracellular matrix in vertebrates. Reaction of *N*-2-chlorobenzyl-L-alanine with sulfonyl chlorides, arylsulfonyl isocyanate, aryl isocyanates or benzoyl isothiocyanate afforded the new derivatives which were subsequently converted to the corresponding hydroxamates. These latter derivatives were 100–500 times more inhibitory against ChC as compared to the corresponding carboxylic acids. Best substitutions for obtaining high affinity inhibitors, involved hydrophobic moieties at $S_{3'}$, such as perfluoroalkylsulfonyl-; substituted-arylsulfonyl (pentafluoro-phenylsulfonyl; 3- and 4-carboxy-phenylsulfonyl-; 3-trifluoromethyl-phenylsulfonyl; or 1- and 2-naphthyl, etc.) moieties among others. 2-Chlorobenzyl moieties at $P_{2'}$ seem to be a valid variant of the benzyl group, for obtaining tight-binding collagenase inhibitors. This is the first study reporting nanomolar affinity ChC inhibitors of the sulfonamide type.

Experimental

Melting points (mp): 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 (±0.4% of the theoretical values, calculated for the proposed formulas—data not shown): 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). Preparative HPLC was performed on a Dynamax-60A column (25×250 mM), with a Beckman EM-1760 instrument. The detection wavelength was 254 nM.

Amino acids (L-Ala), sulfonyl chlorides, arylsulfonyl isocyanates, aryl isocyanates, benzoyl isothiocyanate, triethylamine, carbodiimides, hydroxylamine, 2-chlorobenzyl chloride, and other reagents/solvents used in the syntheses were commercially available compounds (from Sigma, Fluka, Acros or Aldrich).

Preparation of *N*-2-chlorobenzyl-L-alanine (3). An amount of 9 g (0.10 M) of L-Ala (**2**) and the stoichiometric amount of 2-chlorobenzyl chloride (16.1 g, 12.6 mL) were suspended/dissolved in 50 mL of anhydrous acetonitrile and the equivalent amount of triethyl amine (0.10 mM, 14.7 mL) was added. The reaction mixture was heated at reflux for 20 h, then the solvent was evaporated in vacuo. The obtained reaction mixture was taken in 50 mL of water, the pH was brought to 7 with citric acid, and the crude product (**3**) precipitated by leaving the mixture overnight at 4 °C. Recrystallization from ethanol afforded the pure title compound in almost quantitative yield.

General procedure for the preparation of *N*-2-chlorobenzyl-alkyl/arylsulfonyl alanines A1–A34. An amount of 2.13 g (10 mmol) of *N*-2-chlorobenzyl-L-alanine (**3**) and 10 mMol of sulfonyl chloride (**4**) were suspended/dissolved in 100 mL of acetone + 25 mL of water. The stoichiometric amount (10 mmol) of base ($NaHCO_3$; $KHCO_3$, NaOH or Et_3N) dissolved in a small amount (20 mL) of water was added and the mixture stirred at room temperature for 4–10 h (TLC control). The solvent was evaporated, the reaction mixture was retaken in 100 mL of water and the crude product extracted in ethyl acetate. After evaporation of the solvent, the compounds **A1**–**A34** were recrystallized from EtOH or MeOH. Yields were around 75–90%.

General procedure for the preparation of compounds B1–B34, D1–D4, F1–F6, H1–H3 and J1. An amount of 5 mM of carboxylic acid derivative **A1–A34, C1–C4, E1–E6, G1–G3** or **I1** was dissolved/suspended in 50 mL of anhydrous acetonitrile or acetone, and treated with 420 mg (6 mM) of hydroxylamine·HCl and 1.10 g (6 mM) of EDCI. HCl or di-isopropyl-carbodiimide. The reaction mixture was magnetically stirred at room temperature for 15 min, then 180 μL (12 mM) of triethylamine were added and stirring was continued for 12 h at 4 °C. The solvent was evaporated in vacuo and the residue taken up in ethyl acetate (5 mL), poured into a 5% solution of sodium bicarbonate (5 mL) and extracted with ethyl acetate. The combined organic layers were dried over sodium sulfate and filtered, and the solvent removed in vacuo. Preparative HPLC (Dynamax-60A column (25×250 mM); 90% acetonitrile/10% methanol; flow rate of 30 mL/min) afforded the pure hydroxamic acids.

General procedure for the preparation of compounds C1–C4, E1–E6 and I1. An amount of 2.13 g (10 mmol) of *N*-2-chlorobenzyl-alanine (**3**) and the stoichiometric amount of arylsulfonyl isocyanate (**5**); aryl isocyanate (**6**) or benzoyisothiocyanate were suspended in 50 mL of anhydrous acetonitrile and 150 μL (10 mM) of triethylamine were added. The reaction mixture was either stirred at room temperature (in the case of derivatives prepared from **5**) or refluxed (for the other two types of derivatives) for 2–6 h. The solvent was evaporated and the reaction mixture worked up as described above. The new compounds were recrystallized from ethanol. Yields were almost quantitative.

General procedure for the preparation of compounds G1–G3. The general procedure described above for the preparation of compounds **A1**–**A34** has been followed, except that arylsulfenyl halides were used instead of alkyl/arylsulfonyl halides. The yields in the title sulfenamides were around 75%.

The new compounds were characterized by ^1H and ^{13}C NMR spectroscopy and elemental analysis. Data for a representative compound of each series is provided below.

***N*-4-Toluenesulfonyl-*N*-2-chlorobenzyl-L-alanine A13.** White crystals, mp 197–199 °C; ^1H NMR (DMSO-d_6),

δ, ppm: 1.52 (d, $^3J_{HH}=6.5$, 3H, $CHCH_3$ of Ala), 2.53 (s, 3H, $CH_3C_6H_4$), 3.79 (s, 2H, CH_2 of benzyl); 3.90 (q, 1H, CH of Ala); 7.22-7.59 (m, 6H, H_{ortho} of $CH_3C_6H_4$ and H_{arom} of 2-Cl-C_6H_4), 7.92 (d, $^3J_{HH}=8.1$, 2H, H_{meta} of $CH_3C_6H_4$); 11.54 (br s, 1H, COOH); ^{13}C NMR (DMSO-d_6), δ, ppm: 20.3 (s, $CHCH_3$ of Ala); 26.1 (s, $CH_3C_6H_4$), 34.5 (s, $CHCH_3$ of Ala); 43.5 (s, CH_2 of benzyl), 127.2 (s, C-5 of 2-Cl-C_6H_4); 129.7 (C-4 of 2-Cl-C_6H_4); 129.9 (C-3 of 2-Cl-C_6H_4); 130.2 (s, C_{meta} of $CH_3C_6H_4$), 130.8 (C-6 of 2-Cl-C_6H_4); 134.2 (C-2 of 2-Cl-C_6H_4); 135.0 (C-1 of 2-Cl-C_6H_4); 135.7 (s, C_{ortho} of $CH_3C_6H_4$), 145.5 (s, C_{ipso} of $CH_3C_6H_4$), 148.8 (s, C_{para} of $CH_3C_6H_4$), 177.7 (s, CO_2H). $C_{17}H_{18}ClNO_4S$ (360.85); calcd. C, 55.10; H, 4.93; N, 3.81%; found: C, 55.16; H, 5.09; N, 3.75%.

***N*-4-Toluenesulfonyl-*N*-2-chlorobenzyl-L-alanine hydroxamate B13.** White crystals, mp 211–221 °C; ^1H NMR (DMSO-d_6), δ, ppm: 1.54 (d, $^3J_{HH}=6.5$, 3H, $CHCH_3$ of Ala), 2.62 (s, 3H, $CH_3C_6H_4$), 3.79 (s, 2H, CH_2 of benzyl); 3.93 (q, 1H, CH of Ala); 7.24–7.65 (m, 6H, H_{ortho} of $CH_3C_6H_4$ and H_{arom} of 2-Cl-C_6H_4), 8.06 (d, $^3J_{HH}=8.1$, 2H, H_{meta} of $CH_3C_6H_4$); 8.73 (br s, 1H, NHOH); 10.52 (br s, 1H, NHOH); ^{13}C NMR (DMSO-d_6), δ, ppm: 20.4 (s, $CHCH_3$ of Ala); 26.5 (s, $CH_3C_6H_4$), 34.4 (s, $CHCH_3$ of Ala); 44.1 (s, CH_2 of benzyl), 127.2 (s, C-5 of 2-Cl-C_6H_4); 129.7 (C-4 of 2-Cl-C_6H_4); 129.9 (C-3 of 2-Cl-C_6H_4); 130.6 (s, C_{meta} of $CH_3C_6H_4$), 130.8 (C-6 of 2-Cl-C_6H_4); 134.2 (C-2 of 2-Cl-C_6H_4); 135.0 (C-1 of 2-Cl-C_6H_4); 135.3 (s, C_{ortho} of $CH_3C_6H_4$), 145.2 (s, C_{ipso} of $CH_3C_6H_4$), 148.0 (s, C_{para} of $CH_3C_6H_4$), 174.9 (s, CONHOH). $C_{17}H_{19}ClN_2O_4S$ 8382.87) calcd. C, 53.33; H, 5.00; N, 7.32%; found: C, 53.24; H, 5.07; N, 7.25%.

***N*-4-Toluenesulfonylureido-*N*-2-chlorobenzyl-L-alanine C3.** White crystals, mp 207–209 °C; ^1H NMR (DMSO-d_6), δ, ppm: 1.51 (d, $^3J_{HH}=6.5$, 3H, $CHCH_3$ of Ala), 2.60 (s, 3H, $CH_3C_6H_4$), 3.77 (s, 2H, CH_2 of benzyl); 3.95 (q, 1H, CH of Ala); 7.12–7.68 (m, 6H, H_{ortho} of $CH_3C_6H_4$ and H_{arom} of 2-Cl-C_6H_4), 7.99 (d, $^3J_{HH}=8.1$, 2H, H_{meta} of $CH_3C_6H_4$); 8.23 (br s, 2H, NHCONH); 11.70 (br s, 1H, COOH); ^{13}C NMR (DMSO-d_6), δ, ppm: 20.3 (s, $CHCH_3$ of Ala); 26.6 (s, $CH_3C_6H_4$), 34.5 (s, $CHCH_3$ of Ala); 43.3 (s, CH_2 of benzyl), 127.2 (s, C-5 of 2-Cl–C_6H_4); 129.7 (C-4 of 2-Cl–C_6H_4); 129.9 (C-3 of 2-Cl–C_6H_4); 130.4 (s, C_{meta} of $CH_3C_6H_4$), 130.8 (C-6 of 2-Cl-C_6H_4); 132.3 (s, NHCONH), 134.2 (C-2 of 2-Cl–C_6H_4); 135.2 (C-1 of 2-Cl–C_6H_4); 135.6 (s, C_{ortho} of $CH_3C_6H_4$), 145.1 (s, C_{ipso} of $CH_3C_6H_4$), 148.5 (s, C_{para} of $CH_3C_6H_4$), 177.2 (s, CO_2H). $C_{18}H_{19}ClN_2O_5S$ (410.88) calcd. C, 52.62; H, 4.66; N, 6.82%; found: C, 52.49; H, 4.51; N, 6.73%.

***N*-4-Toluenesulfonylureido-*N*-2-chlorobenzyl-L-alanine hydroxamate D3.** White crystals, mp 221–222 °C; ^1H NMR DMSO-d_6), δ, ppm: 1.54 (d, $^3J_{HH}=6.5$, 3H, $CHCH_3$ of Ala), 2.64 (s, 3H, $CH_3C_6H_4$), 3.79 (s, 2H, CH_2 of benzyl); 3.90 (q, 1H, CH of Ala); 7.11–7.62 (m, 6H, H_{ortho} of $CH_3C_6H_4$ and H_{arom} of 2-Cl-C_6H_4), 7.97 (d, $^3J_{HH}=8.1$, 2H, H_{meta} of $CH_3C_6H_4$); 8.23 (br s, 2H, NHCONH); 8.78 (br s, 1H, NHOH); 10.57 (br s, 1H, NHOH); ^{13}C NMR (DMSO-d_6), δ, ppm: 20.5 (s, $CHCH_3$ of Ala); 26.3 (s, $CH_3C_6H_4$), 34.7 (s, $CHCH_3$ of Ala); 44.2 (s, CH_2 of benzyl), 127.2 (s, C-5 of 2-Cl–C_6H_4); 129.7 (C-4 of 2-Cl–C_6H_4); 129.9 (C-3 of 2-Cl–C_6H_4); 130.4 (s, C_{meta} of $CH_3C_6H_4$), 130.8 (C-6 of 2-Cl–C_6H_4); 132.7 (s, NHCONH), 134.2 (C-2 of 2-Cl–C_6H_4); 135.2 (C-1 of 2-Cl–C_6H_4); 135.5 (s, C_{ortho} of $CH_3C_6H_4$), 145.6 (s, C_{ipso} of $CH_3C_6H_4$), 148.5 (s, C_{para} of $CH_3C_6H_4$), 174.7 (s, CONHOH). $C_{18}H_{20}Cl_3O_5S$ (425.89); calcd.: C, 50.76; H, 4.73; N, 9.87%; found: C, 50.85; H, 4.58; N, 9.69%.

***N*-4-Fluorophenylureido-*N*-2-chlorobenzyl-L-alanine E1.** White crystals, mp 176–177 °C; ^1H NMR (DMSO-d_6), δ, ppm: 1.50 (d, $^3J_{HH}=6.5$, 3H, $CHCH_3$ of Ala), 3.78 (s, 2H, CH_2 of benzyl); 3.92 (q, 1H, CH of Ala); 7.11–7.67 (m, 6H, H_{ortho} of 4-FC_6H_4 and H_{arom} of 2-Cl-C_6H_4), 7.95 (d, $^3J_{HH}=8.1$, 2H, H_{meta} of 4-FC_6H_4); 8.13 (br s, 2H, NHCONH); 11.42 (br s, 1H, COOH); ^{13}C NMR (DMSO-d_6), δ, ppm: 20.1 (s, $CHCH_3$ of Ala); 34.5 (s, $CHCH_3$ of Ala); 43.5 (s, CH_2 of benzyl), 127.2 (s, C-5 of 2-Cl-C_6H_4); 129.7 (C-4 of 2-Cl–C_6H_4); 129.9 (C-3 of 2-Cl–C_6H_4); 130.0 (s, C_{meta} of FC_6H_4), 130.8 (C-6 of 2-Cl–C_6H_4); 132.3 (s, NHCONH), 134.2 (C-2 of 2-Cl–C_6H_4); 135.1 (s, C_{ortho} of FC_6H_4), 135.3 (C-1 of 2-Cl–C_6H_4); 148.5 (s, C_{ipso} of FC_6H_4), 149.5 (s, C_{para} of FC_6H_4), 177.5 (s, CO_2H). $C_{17}H_{16}ClFN_2O_3$ (350.78); calcd.: C, 58.21; H, 4.60; N, 7.99%; found: C, 58.42; H, 4.57; N, 8.00%.

***N*-4-Fluorophenylureido-*N*-2-chlorobenzyl-L-alanine hydroxamate F1.** White crystals, mp 210–221 °C; ^1H NMR (DMSO-d_6), δ, ppm: 1.56 (d, $^3J_{HH}=6.5$, 3H, $CHCH_3$ of Ala), 3.82 (s, 2H, CH_2 of benzyl); 3.93 (q, 1H, CH of Ala); 7.08–7.60 (m, 6H, H_{ortho} of 4-FC_6H_4 and H_{arom} of 2-Cl–C_6H_4), 7.90 (d, $^3J_{HH}=8.1$, 2H, H_{meta} of 4-FC_6H_4); 8.12 (br s, 2H, NHCONH); 8.76 (br s, 1H, NHOH); 10.69 (br s, 1H, NHOH); ^{13}C NMR (DMSO-d_6), δ, ppm: 20.3 (s, $CHCH_3$ of Ala); 34.6 (s, $CHCH_3$ of Ala); 43.7 (s, CH_2 of benzyl), 127.2 (s, C-5 of 2-Cl-C_6H_4); 129.7 (C-4 of 2-Cl-C_6H_4); 129.9 (C-3 of 2-Cl-C_6H_4); 130.0 (s, C_{meta} of FC_6H_4), 130.8 (C-6 of 2-Cl-C_6H_4); 132.1 (s, NHCONH), 134.3 (C-2 of 2-Cl–C_6H_4); 135.1 (s, C_{ortho} of FC_6H_4), 135.3 (C-1 of 2-Cl–C_6H_4); 135.6 (s, C_{ortho} of FC_6H_4), 145.8 (s, C_{ipso} of FC_6H_4), 148.4 (s, C_{para} of FC_6H_4), 174.5 (s, CONHOH). $C_{17}H_{17}ClFN_3O_3$ (365.79); calcd.: C, 55.82; H, 4.68; N, 11.49%; found: C, 55.84; H, 4.76; N, 11.33%.

***N*-4-Nitrophenylsulfenyl-*N*-2-chlorobenzyl-L-alanine G1.** Yellow crystals, mp 216–217 °C; ^1H NMR (DMSO-d_6), δ, ppm: 1.50 (d, $^3J_{HH}=6.5$, 3H, CH_3 of Ala), 3.75 (s, 2H, CH_2 of benzyl); 3.93 (q, 1H, CH of Ala); 6.79 (s, 1H, SNH), 7.13–7.64 (m, 6H, H_{ortho} of $O_2NC_6H_4$ and H_{arom} of 2-Cl–C_6H_4), 8.05 (d, $^3J_{HH}=8.3$, 2H, H_{meta} of $O_2NC_6H_4$); 11.73 (br s, 1H, COOH); ^{13}C NMR (DMSO-d_6), δ, ppm: 20.1 (s, $CHCH_3$ of Ala); 34.4 (s, $CHCH_3$ of Ala); 43.5 (s, CH_2 of benzyl), 127.2 (s, C-5 of 2-Cl–C_6H_4); 129.7 (C-4 of 2-Cl–C_6H_4); 129.9 (C-3 of 2-Cl–C_6H_4); 130.1 (s, C_{meta} of $O_2NC_6H_4$), 130.6 (C-6 of 2-Cl–C_6H_4); 134.3 (C-2 of 2-Cl–C_6H_4); 135.2 (C-1 of 2-Cl–C_6H_4); 135.5 (s, C_{ortho} of $O_2NC_6H_4$), 145.4 (s, C_{ipso} of $O_2NC_6H_4$), 150.8 (s, C_{para} of $O_2NC_6H_4$), 177.7 (s, CO_2H). $C_{16}H_{15}ClN_2O_4S$ (366.83); calcd.: C, 52.39; H, 4.12; N, 7.64; found: C, 52.50; H, 4.42; N, 7.51%.

***N*-4-Nitrophenylsulfenyl-*N*-2-chlorobenzyl-L-alanine hydroxamate H1.** Yellow crystals, mp 214–215 °C; ^{1}H NMR (DMSO-d_6), δ, ppm: 1.55 (d, $^3J_{HH}$ = 6.5, 3H, CH_3 of Ala), 3.75 (s, 2H, CH_2 of benzyl); 3.99 (q, 1H, CH of Ala); 6.76 (s, 1H, SNH), 7.11–7.66 (m, 6H, H_{ortho} of $O_2NC_6H_4$ and H_{arom} of 2-Cl–C_6H_4), 8.17 (d, $^3J_{HH}$ = 8.2, 2H, H_{meta} of $O_2NC_6H_4$); 8.75 (br s, 1H, NHOH); 10.68 (br s, 1H, NHOH); ^{13}C NMR (DMSO-d_6), δ, ppm:, 20.1 (s, $CHCH_3$ of Ala); 34.5 (s, $CHCH_3$ of Ala); 43.6 (s, CH_2 of benzyl), 127.1 (s, C-5 of 2-Cl–C_6H_4); 129.7 (C-4 of 2-Cl–C_6H_4); 129.9 (C-3 of 2-Cl–C_6H_4); 130.2 (s, C_{meta} of $O_2NC_6H_4$), 130.7 (C-6 of 2-Cl–C_6H_4); 134.5 (C-2 of 2-Cl–C_6H_4); 135.2 (C-1 of 2-Cl–C_6H_4); 135.9 (s, C_{ortho} of $O_2NC_6H_4$), 145.6 (s, C_{ipso} of $O_2NC_6H_4$), 150.5 (s, C_{para} of $O_2NC_6H_4$), 174.7 (s, CONHOH). $C_{16}H_{16}ClN_3O_4S$ (381.84); calcd.: C, 50.33; H, 4.22; N, 11.00; found: C, 50.45; H, 4.21; N, 10.93%.

Collagenase type VII (highly purified) and FALGPA were purchased from Sigma-Aldrich (Milano, Italy); 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.[30] 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 $\epsilon_{305} = 24{,}700\ L\ mol^{-1}\ cm^{-1}$ in the above-mentioned reaction buffer.[30] Measurements were made using a Perkin–Elmer spectrophotometer interfaced with a PC. Initial velocities were estimated using the direct linear plot-based procedure reported by van Wart and Steinbrink.[30] K_I-s were then determined according to Dixon, using a linear regression program. The K_I values determined are the means of at least three determinations.

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