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Original article

Protease inhibitors – Part 5. Alkyl/arylsulfonyl- and arylsulfonylureido-/arylureido- glycine hydroxamate inhibitors of *Clostridium histolyticum* collagenase

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Abstract – Reaction of alkyl/arylsulfonyl halides with glycine afforded a series of derivatives which were first *N*-benzylated by treatment with benzyl chloride, and then converted to the corresponding hydroxamic acids with hydroxylamine in the presence of carbodiimide derivatives. Other derivatives were obtained by reaction of *N*-benzyl-glycine with aryl isocyanates, arylsulfonyl isocyanates or benzoyl isothiocyanate, followed by conversion of their COOH group into the CONHOH moiety, as mentioned above. The 90 new compounds reported here were assayed as inhibitors of the *Clostridium histolyticum* collagenase (EC 3.4.24.3), a zinc enzyme which degrades triple helical regions of native collagen. The prepared hydroxamate derivatives were generally 100–500 times more active than the corresponding carboxylates. In the series of synthesized hydroxamates, substitution patterns leading to the best inhibitors were those involving perfluoroalkylsulfonyl- and substituted-arylsulfonyl moieties, such as pentafluorophenylsulfonyl, 3- and 4-carboxyphenylsulfonyl-, 3-trifluoromethyl-phenylsulfonyl or 1- and 2-naphthyl among others. Thus, it seems that similarly to the matrix metalloproteinase (MMP) hydroxamate inhibitors, *Clostridium histolyticum* collagenase inhibitors should incorporate hydrophobic moieties at the $P_{1'}$ and $P_{2'}$ sites, whereas the α-carbon substituent may be a small and compact moiety (such as H, for the Gly derivatives reported here). Such compounds might lead to the design of collagenase inhibitor-based drugs useful as anti-cancer, anti-arthritis or anti-bacterial agents for the treatment of corneal keratitis. © 2000 Éditions scientifiques et médicales Elsevier SAS

collagenase / *Clostridium histolyticum* / glycine hydroxamate / zinc metalloproteinase / sulfonamide

1. Introduction

Amino acid and oligopeptide hydroxamates were reported to act as powerful inhibitors for a large number of metallo-enzymes important as targets for the drug design, such as the matrix metalloproteinases, MMP's [1–4], thermolysin and ellastase [5], leucine aminopeptidase [6], carboxypeptidase A [7], leukotriene A4 hydrolase [8], angiotensin I-converting enzyme [9], neurotensin-degrading enzymes [10], endothelin-converting enzyme [11], or UDP-3-O-(R-3-hydroxymyristoyl)-N-acetylglucosamine deacetylase [12–14] among others. Some of these derivatives possess strong antibacterial activity, since by inhibiting some of the above mentioned enzymes they interfere with lipid A biosynthesis and inhibit the growth of Gram-negative bacteria [12–14]. Other compounds of this type were reported to act as anti-HIV agents in vitro [15]. 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 [16–20] or anti-arthritis [2, 21–23] drugs. The 20 MMP's presently known [2, 24] are involved in tissue remodelling connected with tumour invasion and joint destruction [1, 2, 17–21]. 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 [1, 2, 20–23]. The same situation is not true for the inhibitors of other collagenases, such as the enzyme isolated from *Clostridium histolyticum* [25–27], which were much less

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investigated. This collagenase (EC 3.4.24.3) is a 116 kDa protein belonging to the M31 metalloproteinase family [24], which is able to hydrolyse triple helical regions of collagen under physiological conditions, as well as an entire range of synthetic peptide substrates [25–27]. 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, [26, 27], 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 [28].

Similarly to the vertebrate MMP's [2, 24], *Clostridium 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 [29, 30] (in the case of the vertebrate MMP's the zinc ion is co-ordinated by three histidines, His 218, His 222 and His 228), and a water molecule/hydroxide ion acting as a nucleophile in the hydrolytic scission [1–4]. Similarly to the MMP's, ChC is also a multiunit protein, consisting of four segments, S1, S2a, S2b and S3 [30], with S1 incorporating the catalytic domain. Although the two types of collagenases mentioned above (the MMP's type and the bacterial ChC) are relatively different, it is generally considered that their mechanism of action for the hydrolysis of proteins and synthetic substrates is quite similar [1–4, 24, 29, 30]. Thus, we hypothesized that amino acid hydroxamates and some of their derivatives which strongly inhibit MMP's (collagenases, gelatinases, etc.) would also act as potent ChC inhibitors. Our interest in this type of enzyme inhibitor is related to the development of pharmacological agents for the treatment of bacterial corneal keratitis [31], a condition leading to serious complications for which efficient cures are difficult to envisage [32]. It was in fact recently reported [31] that collagen shields applied to the cornea of patients with bacterial keratitis degrade rapidly, due to the collagenases secreted by the pathogen bacterial species, but these shields also protect to some extent the corneal collagen degradation and thus the ocular surface [31, 33–35]. The use of such a shield impregnated with an antibiotic agent specific for the collagen-degrading bacteria would thus have a double benefit for the patient: 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 possess an augmented stability, as its degradation by the secreted collagenases would be delayed, promoting/accelerating in this way the healing of the wound.

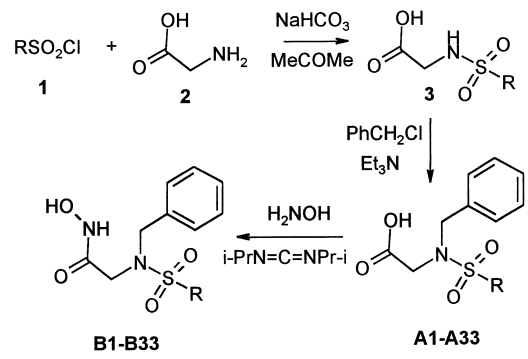

Figure 1. Synthesis of compounds **A1**–**A33** and **B1**–**B33**.

In this paper we report the preparation of a series of ChC inhibitors incorporating alkyl/arylsulfonamido-glycine hydroxamate as well as arylsulfonylureido-/arylureido-glycine hydroxamate moieties in their molecule. Some of the new compounds, assayed for the inhibition of purified ChC, showed nanomolar affinity for the enzyme, behaving as the most potent ChC inhibitors reported up to now.

2. Chemistry

Compounds **A1–A33** were prepared by reaction of alkyl/arylsulfonyl chlorides **1** with glycine **2**, followed by treatment with benzyl chloride, as shown in *figure 1* and *table I*. Conversion of the carboxylic acids **A1–A33** into the corresponding hydroxamates **B1–B33** was done with hydroxylamine and diisopropyl carbodiimide (*figure 1*).

Another series of derivatives (**C1–C4**, **D1–D4** and **E1–E6**, **F1–F6**) was obtained by reaction of arylsulfonylisocyanates **4** or aryl isocyanates **6** with *N*-benzyl-glycine **5**, followed by the conversion of the COOH moiety into the CONHOH one, as described above (*figures 2* and *3*).

Finally, 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 (*figure 4*).

3. Pharmacology

Inhibition data against highly purified ChC type VII, with the newly prepared carboxylic and hydroxamic acids, are shown in *tables I* and *II*. The chromogenic substrate FALGPA (2-furanacryloyl-*L*-Leu-Gly-*L*-Pro-*L*-Ala) was used in the assay, with the spectrophotometric

Table I. Inhibition of ChC with the carboxylic acids **A1–A33** and the corresponding hydroxamates **B1–B33**.

R	Compound	K_i[a] (μM)	Compound	K_i[a] (nM)
CH_3	**A1**	25	**B1**	112
CF_3	**A2**	5	**B2**	52
CCl_3	**A3**	6	**B3**	51
n-C_4F_9-	**A4**	3	**B4**	13
n-C_8F_{17}	**A5**	2	**B5**	9
Me_2N-	**A6**	47	**B6**	94
C_6H_5-	**A7**	24	**B7**	54
$PhCH_2$-	**A8**	19	**B8**	48
4-F-C_6H_4-	**A9**	15	**B9**	51
4-Cl-C_6H_4-	**A10**	14	**B10**	45
4-Br-C_6H_4-	**A11**	14	**B11**	39
4-I-C_6H_4-	**A12**	13	**B12**	30
4-CH_3-C_6H_4-	**A13**	17	**B13**	44
4-O_2N-C_6H_4-	**A14**	6	**B14**	16
3-O_2N-C_6H_4-	**A15**	5	**B15**	15
2-O_2N-C_6H_4-	**A16**	7	**B16**	24
3-Cl-4-O_2N-C_6H_3-	**A17**	3	**B17**	12
4-AcNH-C_6H_4-	**A18**	4	**B18**	13
4-BocNH-C_6H_4-	**A19**	3	**B19**	10
3-BocNH-C_6H_4-	**A20**	2	**B20**	9
C_6F_5-	**A21**	0.7	**B21**	6
3-CF_3-C_6H_4	**A22**	0.7	**B22**	7
2,5-$Cl_2C_6H_3$	**A23**	5	**B23**	21
4-CH_3O-C_6H_4-	**A24**	7	**B24**	27
2,4,6-$(CH_3)_3$-C_6H_2-	**A25**	7	**B25**	15
4-CH_3O-3-BocNH-C_6H_3-	**A26**	3	**B26**	8
2-HO-3,5-Cl_2-C_6H_2-	**A27**	4	**B27**	9
3-HOOC-C_6H_4-	**A28**	5	**B28**	[b]
4-HOOC-C_6H_4-	**A29**	5	**B29**	[b]
1-Naphthyl	**A30**	4	**B30**	11
2-Naphthyl	**A31**	4	**B31**	12
5-Me_2N-1-naphthyl-	**A32**	5	**B32**	13
2-thienyl	**A33**	4	**B33**	16

[a]K_I values were obtained from Dixon plots using a linear regression program [51], from at least three different assays. [b]The C_6H_4–COOH moiety transformed into C_6H_4–CONHOH.

method of van Wart and Steinbrink [25]. Inhibition data with some standard ChC inhibitors reported in the literature are also provided for comparison in *table III*.

4. Results and discussion

Few ChC (or for other bacterial collagenases) inhibitors were reported up to now in the literature [36–42]. As the ChC enzyme catalyses 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 [36].

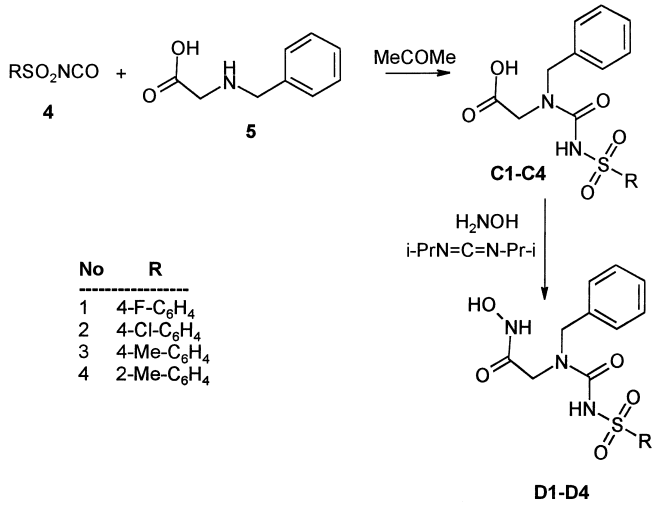

Figure 2. Synthesis of compounds **C1**–**C4** and **D1**–**D4**.

Analogously, the $S_{1'}$, $S_{2'}$ and $S_{3'}$ subsites are also occupied by Gly, Pro and Xaa, respectively [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-amino-hexanoic acid was of 5 nM) [41, 42]. As shown from the

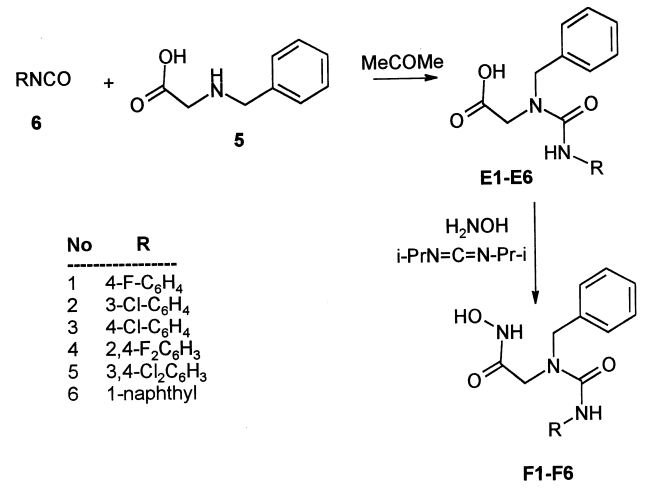

Figure 3. Synthesis of compounds **E1**–**E6** and **F1**–**F6**.

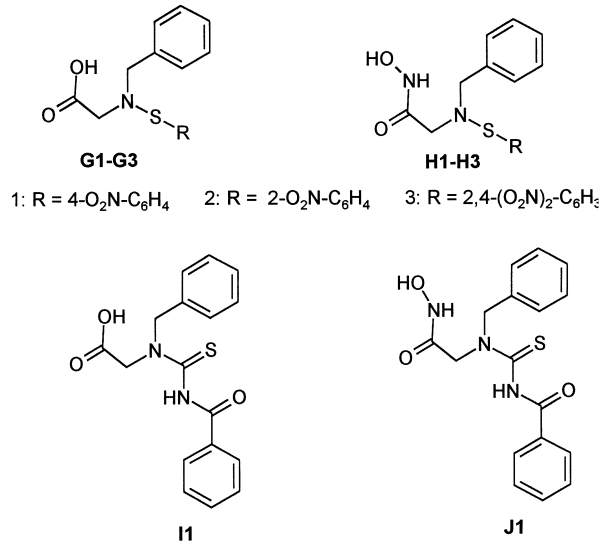

Figure 4. Structures of compounds **G1**–**G3**, **H1**–**H3**, **I1** and **J1**.

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.

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-glycine hydroxamic acids of type **7** recently reported by Parker's group [21], we decided to use such derivatives as lead molecules. In the above mentioned study it was observed that best inhibitors of type **7** (against mouse macrophage metallo-elastase) were those incorporating: i) Gly, Ala or Val as spacers between the zinc-binding function (the hydroxamic acid moiety) and the $P_{1'}$ site; ii) benzyl or isobutyl moieties at $S_{2'}$, and iii) arylsulfonyl moieties at $S_{3'}$ [21] (*figure 5*). It should be noted that a relatively small number of arylsulfonyl moieties were investigated in the above-mentioned study [21], the greatest number of the synthesized inhibitors **7** containing the 4-methoxybenzenesulfonyl moiety. The proposed interaction sites between an inhibitor of type **7** and the active site of the enzyme is shown schematically in *figure 5*.

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

R	Compound	K_i[a] (μM)	Compound	K_i[a] (nM)
4-F-C_6H_4-	**C1**	10	**D1**	21
4-Cl-C_6H_4-	**C2**	7	**D2**	15
4-CH_3-C_6H_4-	**C3**	9	**D3**	16
2-CH_3-C_6H_4-	**C4**	10	**D4**	17
4-F-C_6H_4-	**E1**	12	**F1**	36
3-Cl-C_6H_4-	**E2**	10	**F2**	39
4-Cl-C_6H_4-	**E3**	9	**F3**	34
2,4-F_2-C_6H_3-	**E4**	8	**F4**	30
3,4-$Cl_2C_6H_3$	**E5**	6	**F5**	12
1-Naphthyl	**E6**	5	**F6**	13
4-O_2N-C_6H_4-	**G1**	8	**H1**	17
2-O_2N-C_6H_4-	**G2**	6	**H2**	15
2,4-$(O_2N)_2$-C_6H_3-	**G3**	7	**H3**	10
—	**I1**	0.7	**J1**	6

[a]K_I values were obtained from Dixon plots using a linear regression program [51], from at least three different assays.

Table III. 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 values were obtained from Dixon plots using a linear regression program [51], from at least three different assays. [b]2AX = 2-aminohexanoic acid.

Taking into account the above-mentioned findings that led to strong mouse macrophage metallo-elastase inhibitors [21], 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 hydroxamic acid type) [1, 2]; ii) a small (compact) group in α to it [1, 21]; iii) the already optimized [21] benzyl group at the $S_{2'}$ site, and iv) variable alkyl/arylsulfonyl-, arylsulfonyl-ureido/arylureido-, or arylsulfenyl moieties at $S_{3'}$.

The new compounds reported here were obtained by unexceptional synthetic procedures, as outlined in *figures*

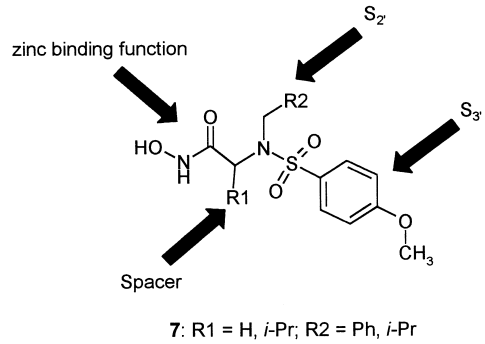

Figure 5. Interaction sites between inhibitor **7** and the enzyme.

1–3. These involved reaction of glycine or *N*-benzyl-glycine 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** and **H**, as well as to the thioureas **I** and **J**.

The following should be noted regarding the ChC inhibition data of *tables I–III* 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) co-ordinating properties of the CONHOH moiety (bidentate binding) as compared to the COOH group (generally monodentate binding to the zinc ion) [1–3]; ii) potent inhibitors were obtained from all classes of hydroxamates investigated here, such as the alkyl/arylsulfonyl-benzyl-Gly derivatives (**B5**, **B20**–**B22**, **B26**, **B27**, **B29**, etc.), the arylsulfonylureas- and arylureas (such as **D2**, **F5** and **F6**), the sulfenamido-benzyl-Gly derivatives (such as **H2** and **H3**) or the thiourea **J1**. Thus, it seems that the $S_{2'}$-binding moiety of the arylsulfonamide type, previously investigated for obtaining MMP inhibitors of type **7** [21], 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** (**1–33**)) best ChC inhibitory properties were correlated with the presence of perfluoroalkylsulfonyl- (**B4**, **B5**), perfluorophenylsulfonyl- (**B21**), 3-trifluoromethyl-phenylsulfonyl- (**B22**), 3-chloro-4-nitro-phenylsulfonyl- (**B17**), 3- or 4-protected-amino-phenylsulfonyl- (**B18–B20** and **B26**), 3- or 4-carboxy-phenylsulfonyl- (**B28** and **B29**) and 1- or 2-naphthylsulfonyl moieties (**B30**–**B32**). All these derivatives possessed inhibition constants in the range of 6–13 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-methoxy-phenyl- or 2-thienyl, substituting the *N*-benzyl-glycine hydroxamate, behaved as medium potency inhibitors, with affinities in the 15–30 nM range (*table I*). The least active sulfonamides were those containing methyl-, trihalomethyl-, dimethylamino-, phenyl- and benzyl moieties (*table I*); 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, medium potency ChC inhibitors. Type **F** ureas behaved similarly, and the sulfenamides of type **H**, except for **F5** and **F6**, as well as

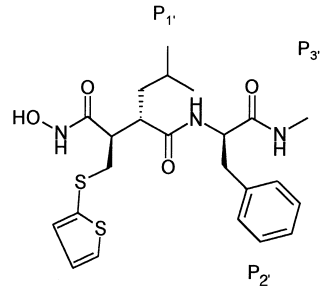

Figure 6. Batimastat **8**.

H2 and **H3**, which are strong inhibitors. A very potent inhibitor is the thiourea derivative **J1** (*table II*). By comparing the data of *tables I–III*, 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 III*, 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 and Tschesche's groups reported [3, 4, 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 the collagenase inhibitor in clinical study, batimastat **8** [50] (*figure 6*).

These studies [3, 4, 47–49] showed that batimastat is bidentately co-ordinated 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 collagenase III inhibitors (in the classification of Babine and Bender [1]) of the sulfonamide type, to which the compounds reported here presumably belong, were much less investigated crystallographically, it is assumed

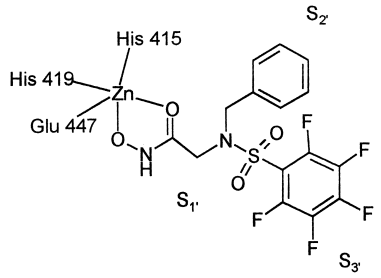

Figure 7. Schematic binding of the inhibitor **B21** within the active site of ChC.

that the general binding mode illustrated above is also valid for them, although some differences were also evidenced [1, 21]. Based on these observations we propose a similar binding mode of the sulfonamide inhibitors reported here to ChC, as shown schematically in *figure 7*.

The inhibitor probably co-ordinates bidentately to the Zn(II) ion of ChC, whereas the hydrophobic moieties from the $P_{2'}$ and $P_{3'}$ sites (benzyl 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 6 nM).

5. Conclusion

We describe here a novel class of strong inhibitors of the zinc protease EC 3.4.24.3, a collagenase from *Clostridium 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 extracellular matrix in vertebrates. Reaction of glycine or *N*-benzyl-glycine with sulfonyl chlorides, arylsulfonyl isocyanate, aryl isocyanates or benzoyl isothiocyanate afforded the glycine derivatives which were subsequently converted into 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) moieties among others. This is the first study reporting nanomolar affinity ChC inhibitors of the sulfonamide type.

6. Experimental section

6.1. Chemistry

Melting points: heating plate microscope (not corrected); IR spectra: KBr pellets, 400–4 000 cm^{-1} Perkin-Elmer 16PC FTIR spectrometer; ^{1}H-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 (Gly, *N*-benzyl-Gly), sulfonyl chlorides, arylsulfonyl isocyanates, aryl isocyanates, benzoyl-isothiocyanate, triethylamine, carbodiimides, hydroxyl-amine, and other reagents used in the syntheses were commercially available compounds (from Sigma, Acros or Aldrich).

6.1.1. General procedure for the preparation of alkyl/arylsulfonyl glycines **3**

An amount of 1.50 g (20 mmol) of glycine **2** and 20 mmol of sulfonyl chloride **1** were suspended/dissolved in 100 mL of acetone + 25 mL of water. The stoichiometric amount (20 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 re-taken in 100 mL of water and the crude **3** extracted in ethyl acetate. After evaporation of the solvent the products were recrystallized from EtOH or MeOH. Yields were around 85–95%.

6.1.2. General procedure for the preparation of derivatives **A1–A33**

An amount of 10 mM of alkyl/arylsulfonyl-Gly **3** and the stoichiometric amount of benzyl chloride were suspended/dissolved in 50 mL of anhydrous acetonitrile and the stoichiometric amount (10 mM) of triethyl amine (10 mM, 1.47 mL) was added. The reaction mixture was heated at reflux for 2 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 carboxylic acids **A1–A33** extracted in ethyl acetate. Recrystallization from methanol/water afforded the pure title compounds in almost quantitative yield.

6.1.3. General procedure for the preparation of compounds **B1–B33**, **D1–D4**, **F1–F6**, **H1–H3** *and* **J1**

An amount of 5 mM of carboxylic acid derivative **A1–A33**, **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.

6.1.4. General procedure for the preparation of compounds **C1–C4**, **E1–E6** *and* **I1**

An amount of 1.65 g (10 mmol) of *N*-benzyl-glycine **5** and the stoichiometric amount of arylsulfonyl isocyanate **4**, aryl isocyanate **6** or benzoylisothiocyanate 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 **4**) 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 at 6.1.1. The new compounds were recrystallized from ethanol. Yields were almost quantitative.

6.1.5. General procedure for the preparation of compounds **G1–G3**

The general procedure described above for the preparation of compounds **A1–A33** was followed, except that *N*-benzyl-glycine **5** was used instead of glycine **2**, and arylsulfenyl halides instead of alkyl/arylsulfonyl halides. The yields in the title sulfenamides were around 90%.

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

6.1.5.1. N-4-Toluenesulfonyl-N-benzyl-glycine **A13**

White crystals, m.p. 192–194 °C; ^{1}H-NMR (DMSO-d_6), δ, ppm: 2.50 (s, 3H, C$\underline{H}_3$C$_6$H$_4$), 3.62 (s, 2H, C$\underline{H}_2$ of Gly), 3.75 (s, 2H, C$\underline{H}_2$ of benzyl); 7.22–7.59 (m, 7H, $\underline{H}_{ortho}$ of $CH_3C_6H_4$ and $\underline{H}_{arom}$ of Ph), 7.97 (d, $^{3}J_{HH}$ = 8.1, 2H, $\underline{H}_{meta}$ of $CH_3C_6H_4$); 11.61 (br s, 1H, COOH); ^{13}C-NMR (DMSO-d_6), δ, ppm: 26.0 (s, $\underline{C}H_3C_6H_4$), 40.4 (s, $\underline{C}H_2$ of Gly), 42.7 (s, $\underline{C}H_2$ of benzyl), 130.1 (s, $\underline{C}_{meta}$ of $CH_3C_6H_4$), 131.8 (s, $\underline{C}_{para}$ of Ph), 133.6 (s, $\underline{C}_{meta}$ of Ph), 134.5 (s, $\underline{C}_{ortho}$ of Ph), 135.0 (s, $\underline{C}_{ortho}$ of $CH_3C_6H_4$), 141.5 (s, $\underline{C}_{ipso}$ of Ph), 145.0 (s, $\underline{C}_{ipso}$ of $CH_3C_6H_4$), 148.6 (s, $\underline{C}_{para}$ of $CH_3C_6H_4$), 177.3 (s, $\underline{C}O_2H$). Anal. $C_{16}H_{17}NO_4S$ (C, H, N).

6.1.5.2. N-4-Toluenesulfonyl-N-benzyl-glycine hydroxamate **B13**

White crystals, m.p. 233–235 °C; ^{1}H-NMR (DMSO-d_6), δ, ppm: 2.62 (s, 3H, C$\underline{H}_3$C$_6$H$_4$), 3.71 (s, 2H, C$\underline{H}_2$ of Gly), 3.77 (s, 2H, C$\underline{H}_2$ of benzyl); 7.24–7.66 (m, 7H, $\underline{H}_{ortho}$ of $CH_3C_6H_4$ and $\underline{H}_{arom}$ of Ph), 8.04 (d, $^{3}J_{HH}$ = 8.1, 2H, H_{meta} of $CH_3C_6H_4$); 8.70 (br s, 1H, N$\underline{H}$OH); 10.48 (br s, 1H, NHO$\underline{H}$); ^{13}C-NMR (DMSO-d_6), δ, ppm: 26.5 (s, $\underline{C}H_3C_6H_4$), 40.6 (s, $\underline{C}H_2$ of Gly), 42.1 (s, $\underline{C}H_2$ of benzyl), 130.7 (s, $\underline{C}_{meta}$ of $CH_3C_6H_4$), 131.8 (s, $\underline{C}_{para}$ of Ph), 133.5 (s, $\underline{C}_{meta}$ of Ph), 134.8 (s, $\underline{C}_{ortho}$ of Ph), 135.6 (s, $\underline{C}_{ortho}$ of $CH_3C_6H_4$), 141.5 (s, $\underline{C}_{ipso}$ of Ph), 145.3 (s, $\underline{C}_{ipso}$ of $CH_3C_6H_4$), 148.6 (s, $\underline{C}_{para}$ of $CH_3C_6H_4$), 174.0 (s, $\underline{C}$ONHOH). Anal. $C_{16}H_{18}N_2O_4S$ (C, H, N).

6.1.5.3. N-4-Toluenesulfonylureido-N-benzyl-glycine **C3**

White crystals, m.p. 217–218 °C; ^{1}H-NMR (DMSO-d_6), δ, ppm: 2.60 (s, 3H, C$\underline{H}_3$C$_6$H$_4$), 3.67 (s, 2H, C$\underline{H}_2$ of Gly), 3.75 (s, 2H, C$\underline{H}_2$ of benzyl); 7.29–7.58 (m, 7H, $\underline{H}_{ortho}$ of $CH_3C_6H_4$ and $\underline{H}_{arom}$ of Ph), 7.99 (d, $^{3}J_{HH}$ = 8.1, 2H, $\underline{H}_{meta}$ of $CH_3C_6H_4$); 8.24 (br s, 2H, NHCONH); 11.76 (br s, 1H, COOH); ^{13}C-NMR (DMSO-d_6), δ, ppm: 26.1 (s, $\underline{C}H_3C_6H_4$), 40.8 (s, $\underline{C}H_2$ of Gly), 42.3 (s, $\underline{C}H_2$ of benzyl), 130.9 (s, $\underline{C}_{meta}$ of $CH_3C_6H_4$), 131.5 (s, $\underline{C}_{para}$ of Ph), 132.4 (s, NH$\underline{C}$ONH), 133.7 (s, $\underline{C}_{meta}$ of Ph), 134.6 (s, $\underline{C}_{ortho}$ of Ph), 135.0 (s, $\underline{C}_{ortho}$ of $CH_3C_6H_4$), 141.5 (s, $\underline{C}_{ipso}$ of Ph), 145.0 (s, $\underline{C}_{ipso}$ of $CH_3C_6H_4$), 148.6 (s, $\underline{C}_{para}$ of $CH_3C_6H_4$), 177.3 (s, $\underline{C}O_2H$). Anal. $C_{17}H_{19}N_3O_5S$ (C, H, N).

6.1.5.4. N-4-Toluenesulfonylureido-N-benzyl-glycine hydroxamate **D3**

White crystals, m.p. 279–281 °C; ^{1}H-NMR (DMSO-d_6), δ, ppm: 2.63 (s, 3H, C$\underline{H}_3$C$_6$H$_4$), 3.69 (s, 2H, C$\underline{H}_2$ of Gly), 3.75 (s, 2H, C$\underline{H}_2$ of benzyl); 7.29–7.62 (m, 7H, $\underline{H}_{ortho}$ of $CH_3C_6H_4$ and $\underline{H}_{arom}$ of Ph), 7.99 (d, $^{3}J_{HH}$ = 8.1, 2H, $\underline{H}_{meta}$ of $CH_3C_6H_4$); 8.24 (br s, 2H, NHCONH); 8.76 (br s, 1H, N$\underline{H}$OH); 10.54 (br s, 1H, NHO$\underline{H}$); ^{13}C-NMR (DMSO-d_6), δ, ppm: 26.3 (s, $\underline{C}H_3C_6H_4$), 40.9 (s, $\underline{C}H_2$ of Gly), 42.2 (s, $\underline{C}H_2$ of benzyl), 130.7 (s, $\underline{C}_{meta}$ of $CH_3C_6H_4$), 131.6 (s, $\underline{C}_{para}$ of Ph), 132.4 (s, NH$\underline{C}$ONH), 133.9 (s, $\underline{C}_{meta}$ of Ph), 134.7 (s, $\underline{C}_{ortho}$ of Ph), 135.0 (s, $\underline{C}_{ortho}$ of $CH_3C_6H_4$), 141.5 (s, $\underline{C}_{ipso}$ of Ph), 145.0 (s, $\underline{C}_{ipso}$ of $CH_3C_6H_4$), 148.6 (s, $\underline{C}_{para}$ of $CH_3C_6H_4$), 174.6 (s, $\underline{C}$ONHOH). Anal. $C_{17}H_{20}N_4O_5S$ (C, H, N).

6.1.5.5. N-4-Fluorophenylureido-N-benzyl-glycine **E1**

White crystals, m.p. 135–136 °C; ^{1}H-NMR (DMSO-d_6), δ, ppm: 3.60 (s, 2H, C$\underline{H}_2$ of Gly), 3.73 (s, 2H, C$\underline{H}_2$ of benzyl); 7.21–7.54 (m, 7H, $\underline{H}_{ortho}$ of FC_6H_4 and $\underline{H}_{arom}$ of Ph), 7.94 (d, $^3J_{HH}$ = 8.0, 2H, $\underline{H}_{meta}$ of FC_6H_4); 8.05 (br s, 2H, NHCONH); 11.44 (br s, 1H, COOH); ^{13}C-NMR (DMSO-d_6), δ, ppm: 40.3 (s, $\underline{C}H_2$ of Gly), 42.6 (s, $\underline{C}H_2$ of benzyl), 130.1 (s, $\underline{C}_{meta}$ of FC_6H_4), 131.6 (s, $\underline{C}_{para}$ of Ph), 132.0 (s, NH$\underline{C}$ONH), 133.9 (s, $\underline{C}_{meta}$ of Ph), 134.5 (s, $\underline{C}_{ortho}$ of Ph), 135.2 (s, $\underline{C}_{ortho}$ of FC_6H_4), 141.4 (s, $\underline{C}_{ipso}$ of Ph), 148.9 (s, $\underline{C}_{ipso}$ of FC_6H_4), 149.7 (s, $\underline{C}_{para}$ of FC_6H_4), 177.8 (s, $\underline{C}O_2H$). Anal. $C_{16}H_{15}FN_2O_3$ (C, H, N).

6.1.5.6. N-4-Fluorophenylureido-N-benzyl-glycine hydroxamate **F1**

White crystals, m.p. 212–213 °C; ^{1}H-NMR (DMSO-d_6), δ, ppm: 3.72 (s, 2H, C$\underline{H}_2$ of Gly), 3.79 (s, 2H, C$\underline{H}_2$ of benzyl); 7.23–7.60 (m, 7H, $\underline{H}_{ortho}$ of FC_6H_4 and $\underline{H}_{arom}$ of Ph), 7.90 (d, $^3J_{HH}$ = 8.2, 2H, $\underline{H}_{meta}$ of FC_6H_4); 8.04 (br s, 2H, NHCONH); 8.71 (br s, 1H, N$\underline{H}$OH); 10.59 (br s, 1H, NHO$\underline{H}$); ^{13}C-NMR (DMSO-d_6), δ, ppm: 40.1 (s, $\underline{C}H_2$ of Gly), 42.4 (s, $\underline{C}H_2$ of benzyl), 130.1 (s, $\underline{C}_{meta}$ of FC_6H_4), 131.3 (s, $\underline{C}_{para}$ of Ph), 131.7 (s, NH$\underline{C}$ONH), 133.8 (s, $\underline{C}_{meta}$ of Ph), 134.7 (s, $\underline{C}_{ortho}$ of Ph), 135.5 (s, $\underline{C}_{ortho}$ of FC_6H_4), 141.7 (s, $\underline{C}_{ipso}$ of Ph), 145.6 (s, $\underline{C}_{ipso}$ of FC_6H_4), 148.9 (s, $\underline{C}_{para}$ of FC_6H_4), 174.8 (s, $\underline{C}$ONHOH). Anal. $C_{16}H_{16}FN_3O_3$ (C, H, N).

6.1.5.7. N-4-Nitrophenylsulfenyl-N-benzyl-glycine **G1**

Yellow crystals, m.p. 175–177 °C; ^{1}H-NMR (DMSO-d_6), δ, ppm: 3.61 (s, 2H, C$\underline{H}_2$ of Gly), 3.75 (s, 2H, C$\underline{H}_2$ of benzyl); 6.75 (s, 1H, SNH), 7.20–7.56 (m, 7H, $\underline{H}_{ortho}$ of $O_2NC_6H_4$ and $\underline{H}_{arom}$ of Ph), 8.05 (d, $^3J_{HH}$ = 8.3, 2H, $\underline{H}_{meta}$ of $O_2NC_6H_4$); 11.68 (br s, 1H, COOH); ^{13}C-NMR (DMSO-d_6), δ, ppm: 40.0 (s, $\underline{C}H_2$ of Gly), 42.8 (s, $\underline{C}H_2$ of benzyl), 130.3 (s, $\underline{C}_{meta}$ of $O_2NC_6H_4$), 131.8 (s, $\underline{C}_{para}$ of Ph), 133.7 (s, $\underline{C}_{meta}$ of Ph), 134.8 (s, $\underline{C}_{ortho}$ of Ph), 135.9 (s, $\underline{C}_{ortho}$ of $O_2NC_6H_4$), 141.2 (s, $\underline{C}_{ipso}$ of Ph), 145.6 (s, $\underline{C}_{ipso}$ of $O_2NC_6H_4$), 150.4 (s, $\underline{C}_{para}$ of $O_2NC_6H_4$), 177.5 (s, $\underline{C}O_2H$). Anal. $C_{15}H_{14}N_2O_4S$ (C, H, N).

6.1.5.8. N-4-Nitrophenylsulfenyl-N-benzyl-glycine hydroxamate **H1**

Yellow crystals, m.p. 182–183 °C; ^{1}H-NMR (DMSO-d_6), δ, ppm: 3.70 (s, 2H, C$\underline{H}_2$ of Gly), 3.79 (s, 2H, C$\underline{H}_2$ of benzyl); 6.75 (s, 1H, SNH), 7.27–7.69 (m, 7H, $\underline{H}_{ortho}$ of $O_2NC_6H_4$ and $\underline{H}_{arom}$ of Ph), 8.09 (d, $^3J_{HH}$ = 8.2, 2H, $\underline{H}_{meta}$ of $O_2NC_6H_4$); 8.77 (br s, 1H, N$\underline{H}$OH); 10.62 (br s, 1H, NHO$\underline{H}$); ^{13}C-NMR (DMSO-d_6), δ, ppm:, 40.5 (s, $\underline{C}H_2$ of Gly), 42.6 (s, $\underline{C}H_2$ of benzyl), 130.6 (s, $\underline{C}_{meta}$ of $O_2NC_6H_4$), 131.3 (s, $\underline{C}_{para}$ of Ph), 133.6 (s, $\underline{C}_{meta}$ of Ph), 134.7 (s, $\underline{C}_{ortho}$ of Ph), 135.9 (s, $\underline{C}_{ortho}$ of $O_2NC_6H_4$), 141.5 (s, $\underline{C}_{ipso}$ of Ph), 148.5 (s, $\underline{C}_{para}$ of $O_2NC_6H_4$), 150.3 (s, $\underline{C}_{ipso}$ of $O_2NC_6H_4$), 174.6 (s, $\underline{C}$ONHOH). Anal. $C_{15}H_{15}N_3O_4S$ (C, H, N).

6.2. Pharmacology

Collagenase type VII (highly purified) and FALGPA were purchased from Sigma Chemical Co. (St. Louis, MO, USA); 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 23 °C, using FALGPA as substrate, by the method of van Wart and Steinbrink [25] 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 L. mol^{-1}. cm^{-1} in the above-mentioned reaction buffer [25]. 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 [25]. K_I's were then determined according to Dixon, using a linear regression program [51]. The K_I values determined are the means of at least three determinations.

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