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PROTEASE INHIBITORS: SYNTHESIS OF L-ALANINE HYDROXAMATE SULFONYLATED DERIVATIVES AS INHIBITORS OF *CLOSTRIDIUM HISTOLYTICUM* COLLAGENASE

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L-alanine hydroxamate derivatives were obtained by reaction of alkyl/arylsulfonyl halides with L-alanine, followed by treatment with benzyl chloride, and conversion of the COOH moiety to the CONHOH group with hydroxylamine in the presence of carbodiimides. Other derivatives were obtained by reaction of *N*-benzyl-alanine with aryl isocyanates, arylsulfonyl isocyanates or benzoyl isothiocyanate, followed by a similar conversion of the COOH to the CONHOH moiety. The obtained compounds were assayed as inhibitors of *Clostridium histolyticum* collagenase, ChC (EC 3.4.24.3), a zinc enzyme which degrades triple helical collagen. The hydroxamate derivatives were generally 100–500 times more active than the corresponding carboxylates. In the series of synthesized derivatives, substitution patterns leading to the most potent ChC inhibitors were those involving perfluoroalkylsulfonyl- and substituted-arylsulfonyl moieties, such as pentafluorophenylsulfonyl, 3- and 4-protected-aminophenylsulfonyl-, 3- and 4-carboxyphenylsulfonyl-, 3-trifluoromethyl-phenylsulfonyl-, or 1- and 2-naphthylsulfonyl 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.

Keywords: Collagenase; *Clostridium histolyticum*; L-alanine hydroxamate; Zinc metalloproteinase; Sulfonamide

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INTRODUCTION

Hydroxamates recently began to be considered as potential therapeutic agents in the treatment of conditions associated with enhanced metalloproteinase (MMP) (mainly collagenase) activity.[1,2] Furthermore, some of these derivatives were also shown to possess *in vivo* anti-tumor activity, without killing normal cells.[3] On the other hand, bacterial corneal keratitis,[4a] a condition leading to serious complications for which efficient cures are not available at the moment,[4b] has also been reported to be associated with highly increased collagenase activity in the ocular tissues.[5]

As amino acid and oligopeptide hydroxamates were reported to act as powerful inhibitors for a large number of metallo-enzymes important as targets in drug design (such as the matrix metalloproteinases) MMPs,[6–9] thermolysin and elastase,[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 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] However 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 MMPs 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 (MMPs 1, 8, 13 and 18), stromelysins 1 and 2 (MMPs 3 and 10, respectively) or the gelatinases A and B (MMPs 2 and 9) have been much investigated recently, in order to develop novel pharmacological agents of the hydroxamate type.[6,7,25–28] The same situation is not true for inhibitors of bacterial collagenases, such as for instance the enzyme isolated from *Clostridium histolyticum*,[30–32] which have been much less investigated. This collagenase (EC 3.4.24.3) is a 116 kDa protein belonging to the M31 MMP family,[29] and is 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 *C. 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

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clostridia, such as *C. perfringens*, which cause human gas gangrene and food poisoning among other effects.[33]

Similarly to the vertebrate MMPs,[7,29] *C. histolyticum* collagenase (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,[34,35] (in the case of the vertebrate MMPs the zinc ion is coordinated by three histidines, His 218, His 222 and His 228), and a water molecule/hydroxide ion acts as nucleophile in the hydrolytic scission.[5–9] Similarly to the MMPs, ChC is also a multidomain protein, consisting of four segments, S1, S2a, S2b and S3,[35] with S1 incorporating the catalytic domain. Although the two types of collagenases mentioned above (the MMPs 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.[5–9,29,34,35]

We propose here that the use of a collagen shield impregnated with 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 increased stability, as its degradation by the secreted collagenases would be delayed, promoting/accelerating in this way the healing of the wound.

Thus, we hypothesized that amino acid hydroxamates and some of their derivatives which strongly inhibit MMPs (collagenases, gelatinases, stromelysins, etc.) would also act as potent ChC inhibitors. In this paper we report the preparation of a series of ChC inhibitors incorporating alkyl/arylsulfonamido-L-alanine hydroxamate as well as arylsulfonylureido-/arylureido-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 some of the best ChC inhibitors reported to date. It is thus quite probable that compounds of the type reported in this paper also inhibit the collagenase activity of bacterial species involved in corneal bacterial keratitis, but the results of the *in vivo* experiments will be reported elsewhere.

MATERIAL AND METHODS

Melting points were determined on a heated plate microscope (not corrected); IR spectra as KBr pellets, over 400–4000 cm^{-1} using a Perkin-Elmer

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16PC FTIR spectrometer; ^{1}H-NMR spectra with a Varian Gemmini 300 apparatus (chemical shifts are expressed as δ values relative to Me_4Si as standard); elemental analysis ($\pm$0.4% of the theoretical values, calculated for the proposed formulas – data not shown) with a 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, and other reagents/solvents used in the syntheses were commercially available compounds (from Sigma, Acros or Aldrich). *N*-Benzyl-Ala was prepared by benzylation of alanine with benzyl chloride.

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General Procedure for the Preparation of Alkyl/Arylsulfonyl Alanines 3

An amount of 1.78 g (20 mmol) of L-alanine **2** and 20 mmol of sulfonyl chloride **1** were suspended/dissolved in a mixture of 100 mL of acetone and 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 monitoring). The solvent was evaporated, the reaction mixture was taken up 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 80–92%.

General Procedure for the Preparation of Derivatives A1–A33

An amount of 10 mM of alkyl/arylsulfonyl-L-Ala **3** and the stoichiometric amount of benzyl chloride were suspended/dissolved in 50 mL of anhydrous acetonitrile and the stoichiometric amount (10 mM) of triethylamine (10 mM, 1.47 mL) was added. The reaction mixture was heated at reflux for 2 h and then the solvent was evaporated *in vacuo*. The residue was taken up in 50 mL of water, adjusted to pH 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.

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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-isopropylcarbodiimide. 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 then evaporated *in vacuo*, the residue taken up in ethyl acetate (5 mL) and the solution, poured into a 5% solution of sodium bicarbonate (5 mL) and extracted with ethyl acetate. The combined organic layers were dried over sodium sulfate, 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 1.79 g (10 mmol) of *N*-benzyl-alanine **5** and the stoichiometric amount of arylsulfonyl isocyanate **4**, 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 **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 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–A33** has been followed, except that *N*-benzyl-Ala **5** was used instead of alanine **2**, and arylsulfenyl halides instead of alkyl/arylsulfonyl halides. The yields in the title sulfenamides were around 75%.

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.

N-4-Toluenesulfonyl-*N*-benzyl-L-alanine **A13**, as white crystals, m.p. 176–7°C; ^{1}H-NMR (DMSO-d_6), δ, ppm: 1.82 (d, $^{3}J_{HH} = 6.5$, 3H, CHCH_3

of Ala), 2.53 (s, 3H, $CH_3C_6H_4$), 3.75 (s, 2H, CH_2 of benzyl), 3.94 (q, 1H, CH of Ala), 7.22–7.59 (m, 7H, H_{ortho} of $CH_3C_6H_4$ and H_{arom} of Ph), 7.97 (d, $^3J_{HH} = 8.1$, 2H, H_{meta} of $CH_3C_6H_4$), 11.61 (br s, 1H, COOH). ^{13}C-NMR (DMSO-d_6), δ, ppm: 22.0 (s, $CHCH_3$ of Ala), 26.1 (s, $CH_3C_6H_4$), 34.5 (s, $CHCH_3$ of Ala), 42.1 (s, CH_2 of benzyl), 130.2 (s, C_{meta} of $CH_3C_6H_4$), 131.9 (s, C_{para} of Ph), 133.4 (s, C_{meta} of Ph), 134.4 (s, C_{ortho} of Ph), 135.7 (s, C_{ortho} of $CH_3C_6H_4$), 141.8 (s, C_{ipso} of Ph), 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). Found: C, 61.10; H, 5.88; N, 4.02. $C_{17}H_{19}NO_4S$ requires: C, 61.24; H, 5.74; N, 4.20%.

N-4-Toluenesulfonyl-*N*-benzyl-L-alanine hydroxamate **B13**, as white crystals, m.p. 217–9°C; ^{1}H-NMR (DMSO-d_6), δ, ppm: 1.82 (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), 7.24–7.60 (m, 7H, H_{ortho} of $CH_3C_6H_4$ and H_{arom} of Ph), 8.02 (d, $^3J_{HH} = 8.1$, 2H, H_{meta} of $CH_3C_6H_4$), 8.70 (br s, 1H, N*H*OH), 10.49 (br s, 1H, NHO*H*). ^{13}C-NMR (DMSO-d_6), δ, ppm: 22.0 (s, $CHCH_3$ of Ala), 26.5 (s, $CH_3C_6H_4$), 34.4 (s, $CHCH_3$ of Ala), 42.1 (s, CH_2 of benzyl), 130.7 (s, C_{meta} of $CH_3C_6H_4$), 131.8 (s, C_{para} of Ph), 133.5 (s, C_{meta} of Ph), 134.8 (s, C_{ortho} of Ph), 135.3 (s, C_{ortho} of $CH_3C_6H_4$), 141.6 (s, C_{ipso} of Ph), 145.2 (s, C_{ipso} of $CH_3C_6H_4$), 148.1 (s, C_{para} of $CH_3C_6H_4$), 174.5 (s, *C*ONHOH). Found: C, 58.76; H, 6.01; N, 8.03. $C_{17}H_{20}N_2O_4S$ requires: C, 58.60; H, 5.79; N, 8.04%.

N-4-Toluenesulfonylureido-*N*-benzyl-L-alanine **C3**, as white crystals, m.p. 180–2°C; ^{1}H-NMR (DMSO-d_6), δ, ppm: 1.82 (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), 7.29–7.58 (m, 7H, H_{ortho} of $CH_3C_6H_4$ and H_{arom} of Ph), 7.99 (d, $^3J_{HH} = 8.1$, 2H, H_{meta} of $CH_3C_6H_4$), 8.25 (br s, 2H, NHCONH), 11.72 (br s, 1H, COOH). ^{13}C-NMR (DMSO-d_6), δ, ppm: 22.3 (s, $CHCH_3$ of Ala), 26.4 (s, $CH_3C_6H_4$), 34.5 (s, $CHCH_3$ of Ala), 42.3 (s, CH_2 of benzyl), 130.4 (s, C_{meta} of $CH_3C_6H_4$), 131.5 (s, C_{para} of Ph), 132.1 (s, NH*C*ONH), 133.6 (s, C_{meta} of Ph), 134.6 (s, C_{ortho} of Ph), 135.0 (s, C_{ortho} of $CH_3C_6H_4$), 141.5 (s, C_{ipso} of Ph), 145.4 (s, C_{ipso} of $CH_3C_6H_4$), 148.7 (s, C_{para} of $CH_3C_6H_4$), 177.6 (s, CO_2H). Found: C, 57.35; H, 5.12; N, 7.28. $C_{18}H_{20}N_2O_5S$ requires: C, 57.43; H, 5.36; N, 7.44%.

N-4-Toluenesulfonylureido-*N*-benzyl-L-alanine hydroxamate **D3**, as white crystals, m.p. 222–3°C; ^{1}H-NMR (DMSO-d_6), δ, ppm: 1.84 (d, $^3J_{HH} = 6.5$, 3H, $CHCH_3$ of Ala), 2.62 (s, 3H, $CH_3C_6H_4$), 3.75 (s, 2H, CH_2 of benzyl), 3.94 (q, 1H, CH of Ala), 7.23–7.61 (m, 7H, H_{ortho} of $CH_3C_6H_4$ and H_{arom} of Ph), 7.98 (d, $^3J_{HH} = 8.1$, 2H, H_{meta} of $CH_3C_6H_4$), 8.26 (br s, 2H, NHCONH), 8.75 (br s, 1H, N*H*OH), 10.54 (br s, 1H, NHO*H*). ^{13}C-NMR (DMSO-d_6), δ, ppm: 22.5 (s, $CHCH_3$ of Ala), 26.3 (s, $CH_3C_6H_4$), 34.6

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(s, $CHCH_3$ of Ala), 42.0 (s, CH_2 of benzyl), 130.5 (s, C_{meta} of $CH_3C_6H_4$), 131.6 (s, C_{para} of Ph), 132.8 (s, NH*C*ONH), 133.9 (s, C_{meta} of Ph), 134.6 (s, C_{ortho} of Ph), 135.3 (s, C_{ortho} of $CH_3C_6H_4$), 141.7 (s, C_{ipso} of Ph), 145.4 (s, C_{ipso} of $CH_3C_6H_4$), 148.5 (s, C_{para} of $CH_3C_6H_4$), 174.8 (s, *C*ONHOH). Found: C, 55.39; H, 5.54; N, 10.69. $C_{18}H_{21}N_3O_5S$ requires: C, 55.23; H, 5.41; N, 10.73%.

N-4-Fluorophenylureido-*N*-benzyl-L-alanine **E1**, as white crystals, m.p. 154–5°C, ^{1}H-NMR (DMSO-d$_6$), δ, ppm: 1.85 (d, $^3J_{HH}=6.5$, 3H, $CHCH_3$ of Ala), 3.73 (s, 2H, CH_2 of benzyl), 3.98 (q, 1H, C*H* of Ala), 7.21–7.54 (m, 7H, H_{ortho} of FC_6H_4 and H_{arom} of Ph), 7.97 (d, $^3J_{HH}=8.1$, 2H, H_{meta} of FC_6H_4), 8.06 (br s, 2H, NHCONH), 11.40 (br s, 1H, COOH). ^{13}C-NMR (DMSO-d$_6$), δ, ppm: 22.1 (s, $CHCH_3$ of Ala), 34.5 (s, $CHCH_3$ of Ala), 42.5 (s, CH_2 of benzyl), 130.0 (s, C_{meta} of FC_6H_4), 131.8 (s, C_{para} of Ph), 132.3 (s, NH*C*ONH), 133.9 (s, C_{meta} of Ph), 134.6 (s, C_{ortho} of Ph), 135.1 (s, C_{ortho} of FC_6H_4), 141.9 (s, C_{ipso} of Ph), 148.5 (s, C_{ipso} of FC_6H_4), 149.5 (s, C_{para} of FC_6H_4), 177.0 (s, CO_2H). Found: C, 64.62; H, 5.37; N, 8.60. $C_{17}H_{17}FN_2O_3$ requires: C, 64.55; H, 5.42; N, 8.86%.

N-4-Fluorophenylureido-*N*-benzyl-L-alanine hydroxamate **F1**, as white crystals, m.p. 221–2°C; ^{1}H-NMR (DMSO-d$_6$), δ, ppm: 1.83 (d, $^3J_{HH}=6.5$, 3H, $CHCH_3$ of Ala), 3.79 (s, 2H, CH_2 of benzyl), 3.99 (q, 1H, C*H* of Ala), 7.19–7.61 (m, 7H, H_{ortho} of FC_6H_4 and H_{arom} of Ph), 7.90 (d, $^3J_{HH}=8.1$, 2H, H_{meta} of FC_6H_4), 8.07 (br s, 2H, NHCONH), 8.75 (br s, 1H, N*H*OH), 10.63 (br s, 1H, NHO*H*). ^{13}C-NMR (DMSO-d$_6$), δ, ppm: 22.0 (s, $CHCH_3$ of Ala), 34.6 (s, $CHCH_3$ of Ala), 42.8 (s, CH_2 of benzyl), 130.0 (s, C_{meta} of FC_6H_4), 131.5 (s, C_{para} of Ph), 131.4 (s, NH*C*ONH), 133.9 (s, C_{meta} of Ph), 134.3 (s, C_{ortho} of Ph), 135.5 (s, C_{ortho} of FC_6H_4), 141.0 (s, C_{ipso} of Ph), 145.8 (s, C_{ipso} of FC_6H_4), 148.4 (s, C_{para} of FC_6H_4), 174.2 (s, *C*ONHOH). Found: C, 61.84; H, 5.52; N, 12.63. $C_{17}H_{18}FN_3O_3$ requires: C, 61.62; H, 5.48; N, 12.68%.

N-4-Nitrophenylsulfenyl-*N*-benzyl-L-alanine **G1**, as yellow crystals, m.p. 177–9°C; ^{1}H-NMR (DMSO-d$_6$), δ, ppm: 1.80 (d, $^3J_{HH}=6.5$, 3H, $CHCH_3$ of Ala), 3.75 (s, 2H, CH_2 of benzyl), 3.96 (q, 1H, C*H* of Ala), 6.78 (s, 1H, SN*H*), 7.20–7.56 (m, 7H, H_{ortho} of $O_2NC_6H_4$ and H_{arom} of Ph), 8.05 (d, $^3J_{HH}=8.3$, 2H, H_{meta} of $O_2NC_6H_4$), 11.68 (br s, 1H, COOH). ^{13}C-NMR (DMSO-d$_6$), δ, ppm: 22.0 (s, $CHCH_3$ of Ala), 34.2 (s, $CHCH_3$ of Ala), 42.7 (s, CH_2 of benzyl), 130.1 (s, C_{meta} of $O_2NC_6H_4$), 131.8 (s, C_{para} of Ph), 133.6 (s, C_{meta} of Ph), 134.3 (s, C_{ortho} of Ph), 135.5 (s, C_{ortho} of $O_2NC_6H_4$), 141.6 (s, C_{ipso} of Ph), 145.7 (s, C_{ipso} of $O_2NC_6H_4$), 150.8 (s, C_{para} of $O_2NC_6H_4$), 177.7 (s, CO_2H). Found: C, 57.76; H, 4.69; N, 8.37. $C_{16}H_{16}N_2O_4S$ requires: C, 57.82; H, 4.85; N, 8.43%.

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N-4-Nitrophenylsulfenyl-*N*-benzyl-L-alanine hydroxamate **H1**, as yellow crystals, m.p. 188–90°C; ^{1}H-NMR (DMSO-d_6), δ, ppm: 1.81 (d, $^3J_{HH}=6.5$, 3H, $CHCH_3$ of Ala), 3.75 (s, 2H, CH_2 of benzyl), 3.99 (q, 1H, CH of Ala), 6.74 (s, 1H, SNH), 7.21–7.66 (m, 7H, H_{ortho} of $O_2NC_6H_4$ and H_{arom} of Ph), 8.12 (d, $^3J_{HH}=8.2$, 2H, H_{meta} of $O_2NC_6H_4$), 8.73 (br s, 1H, $NHOH$), 10.65 (br s, 1H, $NHOH$). ^{13}C-NMR (DMSO-d_6), δ, ppm: 22.1 (s, $CHCH_3$ of Ala), 34.5 (s, $CHCH_3$ of Ala), 42.6 (s, CH_2 of benzyl), 130.4 (s, C_{meta} of $O_2NC_6H_4$), 131.2 (s, C_{para} of Ph), 133.6 (s, C_{meta} of Ph), 134.7 (s, C_{ortho} of Ph), 135.6 (s, C_{ortho} of $O_2NC_6H_4$), 141.4 (s, C_{ipso} of Ph), 148.5 (s, C_{para} of $O_2NC_6H_4$), 150.3 (s, C_{ipso} of $O_2NC_6H_4$), 174.9 (s, $CONHOH$). Found: C, 55.48; H, 5.21; N, 12.10. $C_{16}H_{17}N_3O_4S$ requires: C, 55.32; H, 4.93; N, 12.10%.

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 novel 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 $\varepsilon_{305} = 24{,}700\ L\,mol^{-1}\,cm^{-1}$ in the abovementioned 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 values were then determined according to Dixon, using a linear regression program. The K_I values determined are the means of at least three determinations.

RESULTS AND DISCUSSION

A literature search showed that few inhibitors of ChC (or other bacterial collagenases) had been reported up to now.[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.[36] Analogously, the $S_{1'}$, $S_{2'}$ and $S_{3'}$ subsites are also occupied by Gly, Pro and Xaa, respectively.[36] Thus, many of the

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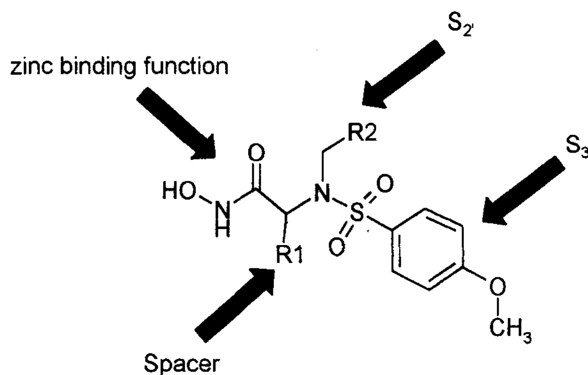
reported inhibitors of ChC are aldehyde or ketone-type substrate analogues, such as Pro_6-Gly-*L*-Pro-GlyH (GlyH = glycine aldehyde), with a K_I of 340 μM;[36] phosphoric and phosphonic amide, such as *iso*-amylphosphonyl-Gly-*L*-Pro-*L*-Ala (K_I of 16 μM);[37] thiols such as HS–CH_2CH_2CO–Pro-Xaa (K_I 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 5 nM).[41,42] As shown from the above data, either the 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.



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

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,[26] we decided to use such derivatives as lead molecules. In the above-mentioned study it was observed that the 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 $P_{2'}$ 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 study,[26] and most of them contained the 4-methoxybenzenesulfonyl moiety. The proposed interaction sites between the inhibitor and the active site of the enzyme is also shown schematically in the formula for the inhibitor **7**.

Considering the above-mentioned findings,[26] we opted in the present study for the inclusion of the following structural elements in the design of

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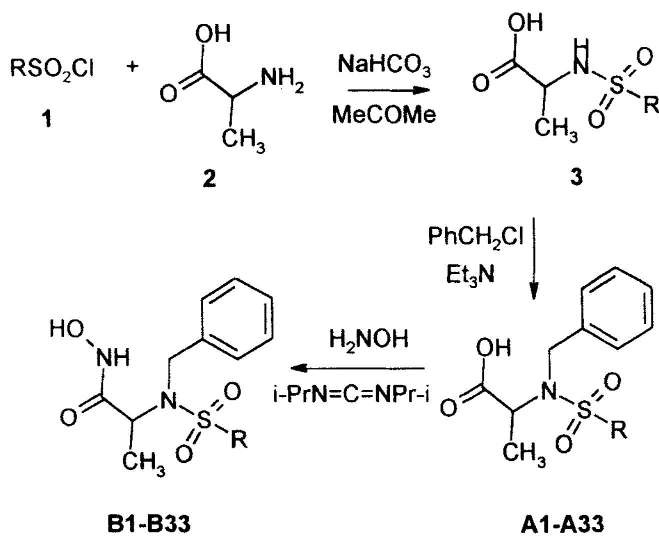
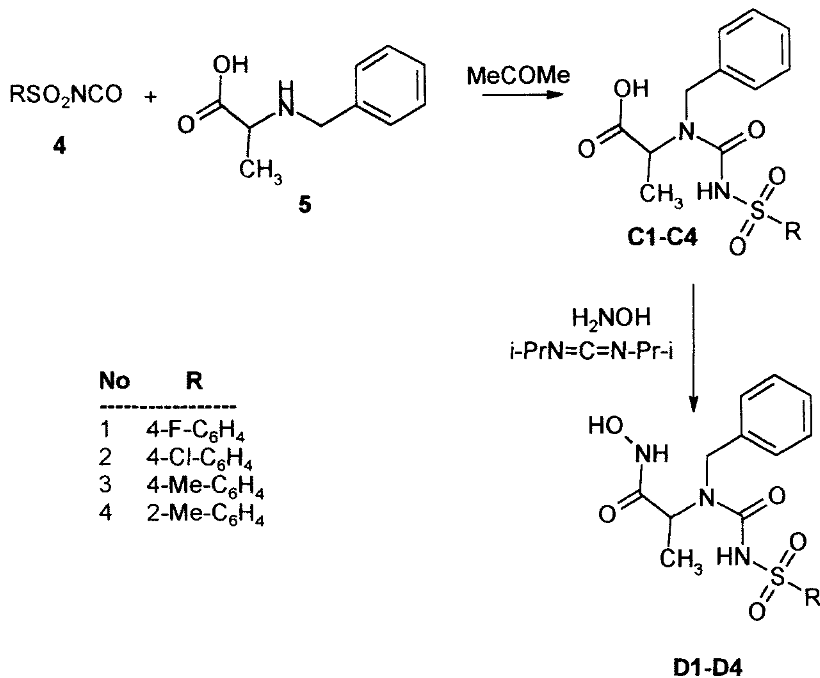


SCHEME 1 Synthesis of **A1–A33** and **B1–B33**.



SCHEME 2 Synthesis of **C1–C4** and **D1–D4**.

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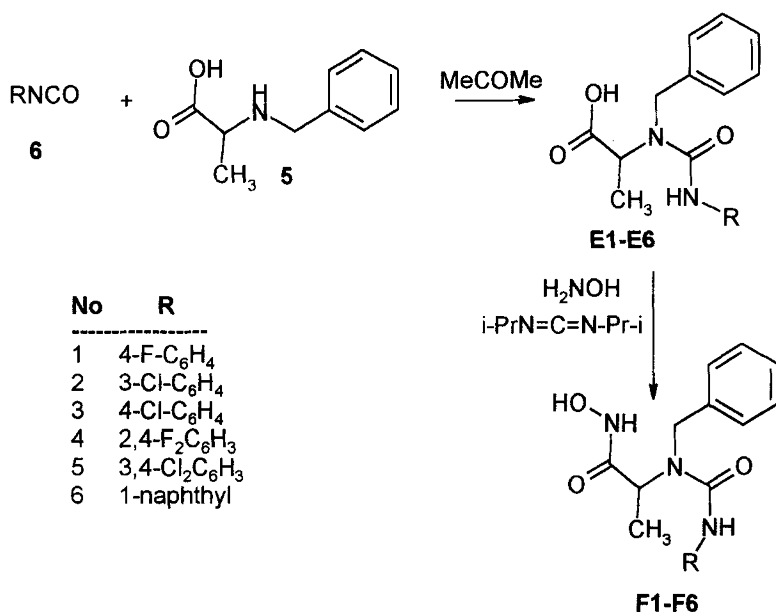
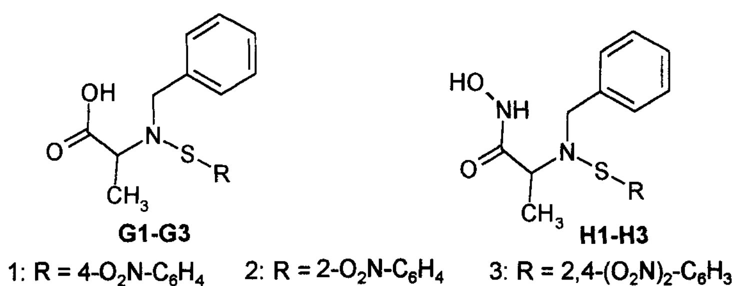


SCHEME 3 Synthesis of **E1–E6** and **F1–F6**.

the ChC inhibitors: (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) the already optimized[26] 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 standard synthetic procedures, as outlined in Schemes 1–3. These involved reaction of L-alanine or *N*-benzyl-L-alanine with alkyl/arylsulfonyl chlorides,[43] aryliso-cyanates,[44] arylsulfonyl-isocyanates[45] or benzoyl-isothiocyanate, followed by conversion of the COOH moiety to the hydroxamate.[46] Related synthetic strategies led to some sulfenamides of type **G**, **H**, as well as to the thioureas **I**, **J**.



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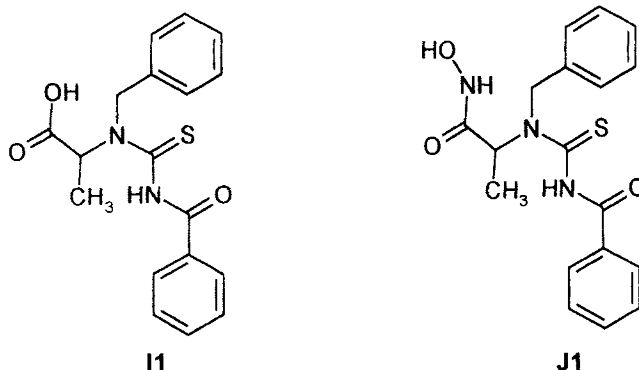

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ChC inhibition data for the new compounds reported here as well as other reported inhibitors are shown in Tables I–III.

The following should be noted regarding ChC inhibition data (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) 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-benzyl-Ala derivatives (**B5**, **B20–B22**, **B26**, **B27**, **B29**, etc.), the arylsulfonylureas- and arylureas (such as **D2**, **F5**, **F6**), the sulfenamido-benzyl-Ala derivatives (such as **H2**, **H3**) or the thiourea **J1**. Thus, it seems that the $S_{2'}$-binding moiety of the arylsulfonamide type, previously investigated for the obtaining of MMP inhibitors of type **7**,[26] can be efficiently substituted by related moieties such as alkylsulfonyl-, arylsulfenyl-, arylsulfonylureido-, arylureido- or benzoylthioureido, without loss of ChC inhibitory properties, (iii) in the subseries of alkyl/arylsulfonamido derivatives (of types **A**, **B** (**1–33**)) the 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-amino-phenylsulfonyl- (**B18–B20**; **B26**), 3- or 4-carboxy-phenylsulfonyl- (**B28**, **B29**) and 1- or 2-naphthylsulfonyl moieties (**B30–B32**). All these derivatives possessed inhibition constants in the range of 5–12 nM against ChC, being among the most potent inhibitors yet reported. A second group of sulfonamide inhibitors, containing moieties such as 4-bromophenyl,

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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**	20	**B1**	103
CF_3	**A2**	3.6	**B2**	48
CCl_3	**A3**	5.5	**B3**	50
n-C_4F_9–	**A4**	3.1	**B4**	12
n-C_8F_{17}	**A5**	1.8	**B5**	7
Me_2N–	**A6**	40	**B6**	68
C_6H_5–	**A7**	22	**B7**	51
$PhCH_2$–	**A8**	17	**B8**	43
4-F–C_6H_4–	**A9**	13	**B9**	45
4-Cl–C_6H_4–	**A10**	13	**B10**	44
4-Br–C_6H_4–	**A11**	11	**B11**	37
4-I–C_6H_4–	**A12**	10	**B12**	32
4-CH_3–C_6H_4–	**A13**	16	**B13**	40
4-O_2N–C_6H_4–	**A14**	5.5	**B14**	15
3-O_2N–C_6H_4–	**A15**	5.2	**B15**	12
2-O_2N–C_6H_4–	**A16**	5.9	**B16**	21
3-Cl-4-O_2N–C_6H_3–	**A17**	3.0	**B17**	11
4-AcNH–C_6H_4–	**A18**	3.6	**B18**	12
4-BocNH–C_6H_4–	**A19**	2.7	**B19**	9
3-BocNH–C_6H_4–	**A20**	2.2	**B20**	7
C_6F_5–	**A21**	0.5	**B21**	6
3-CF_3–C_6H_4–	**A22**	0.6	**B22**	6
2,5-Cl_2–C_6H_3–	**A23**	4.7	**B23**	14
4-CH_3O–C_6H_4–	**A24**	6.2	**B24**	20
2,4,6-$(CH_3)_3$–C_6H_2–	**A25**	6.8	**B25**	19
4-CH_3O–3-BocNH–C_6H_3–	**A26**	3.1	**B26**	7
2-HO–3,5-Cl_2–C_6H_2–	**A27**	3.0	**B27**	8
3-HOOC–C_6H_4–	**A28**	4.5	**B28**[b]	9
4-HOOC–C_6H_4–	**A29**	2.9	**B29**[b]	7
1-Naphthyl	**A30**	3.6	**B30**	10
2-Naphthyl	**A31**	1.4	**B31**	9
5-Me_2N–1-naphthyl–	**A32**	2.5	**B32**	10
2-thienyl	**A33**	2.4	**B33**	11

[a]K_I 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.

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 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 (cf. **D1** with **B9**; **D2** and **B10**, etc.), acting as strong – medium potency ChC inhibitors. Similarly behaved are the ureas of type **F**, the sulfenamides of type **H**, except for **F5** and **F6**, as well as **H2** and **H3**, which are strong inhibitors. A very potent

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TABLE II 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**	8.2	**D1**	18
4-Cl–C_6H_4–	**C2**	6.4	**D2**	15
4-CH_3–C_6H_4–	**C3**	7.1	**D3**	15
2-CH_3–C_6H_4–	**C4**	9.0	**D4**	14
4-F–C_6H_4–	**E1**	10	**F1**	33
3-Cl–C_6H_4–	**E2**	11	**F2**	34
4-Cl–C_6H_4–	**E3**	8.8	**F3**	30
2,4-F_2–C_6H_3–	**E4**	7.3	**F4**	24
3,4-Cl_2–C_6H_3	**E5**	4.8	**F5**	10
1-Naphthyl	**E6**	5.1	**F6**	11
4-O_2N–C_6H_4–	**G1**	6.0	**H1**	15
2-O_2N–C_6H_4–	**G2**	6.3	**H2**	12
2,4-$(O_2N)_2$–C_6H_3–	**G3**	7.1	**H3**	10
	I1	0.5	**J1**	5

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

inhibitor is the thiourea derivative **J1** (Table II). By comparing the data of Tables I–III, it is clear that the compounds reported in the present study are among the best ChC inhibitors obtained to date, since other such derivatives usually had affinities in the micromolar range (except for the phosphonic acid derivative mentioned in Table III, which possessed affinities of the same order of magnitude as those 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 MMPs have been made recently, which might be useful in interpreting our inhibition data. Thus, Bode'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

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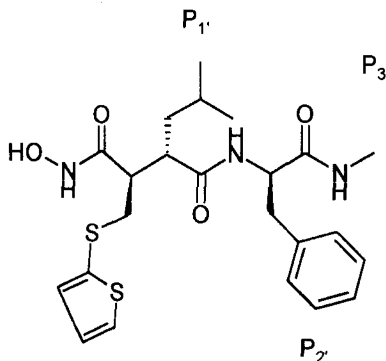
with a K_I of 10 nM by the unique collagenase inhibitor in clinical study, batimastat **8**.[50]



8: Batimastat

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 seen.[6,26] 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 $P_{1'}$ and $P_{2'}$ 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 $K_I = 6$ nM).

In 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 MMPs, related enzymes which

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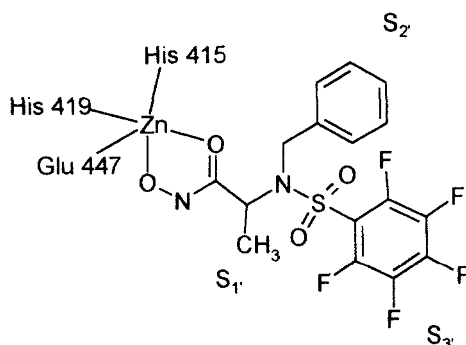

FIGURE 1 Proposed schematic binding of inhibitor **B21** within the active site of ChC.

degrade extracellular matrix in vertebrates. The hydroxamates 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 $S_{2'}$, such as perfluoroalkylsulfonyl-, substituted-arylsulfonyl (pentafluoro-phenylsulfonyl, 3- and 4-carboxy-phenylsulfonyl-, 3-trifluoromethyl-phenylsulfonyl or 1- and 2-naphthylsulfonyl) moieties among others. This is the first study reporting nanomolar affinity ChC inhibitors of the sulfonamide type.

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