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INHIBITION OF HUMAN COLLAGENASES BY SULFUR-BASED SUBSTRATE ANALOGS

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Summary: A series of sulfhydryl and novel sulfur-based substrate-analog inhibitors has been synthesized and tested against human fibroblast and neutrophil collagenases. Absolute stereospecific synthesis of several sulfhydryl inhibitors establishes that it is the diastereomers with the R-configuration of the P'_1 residues, which correspond to the unnatural D-amino acid analogs, that are the most potent inhibitors. The corresponding disulfide, sulfonate, sulfinate, sulfide, sulfoxide and sulfone analogs exhibit widely variable levels of potency, but all less than the sulfhydryl compounds. No correlation between inhibitor potency and any single structural feature of these new compounds is apparent. However, differences in potency can be ascribed to the different affinities of these functional groups for zinc coordination and hydrogen bonding to nearby active site residues.

The metabolic turnover of interstitial collagens in the extracellular matrix of humans is initiated by specific collagenases (EC 3.4.24.7) (1,2). The known human collagenases can be separated into either the fibroblast (3,4) or neutrophil (5,6) types. In addition to the role that these collagenases play in normal collagen turnover, there are many pathological conditions, including rheumatoid arthritis, periodontal disease and inflammation, in which their action gives rise to undesired collagen catabolism (7). Thus, it is of considerable interest to develop specific potent inhibitors of both human fibroblast (HFC) and human neutrophil collagenases (HNC) for potential therapeutic use in the control of these enzymes.

An established means of developing specific proteinase inhibitors is to synthesize substrate analogs in which the scissile peptide bond is replaced by a noncleavable moiety. For zinc proteinases, this approach has involved substitution of the scissile amide bond by

Abbreviations: HFC, human fibroblast collagenase; HNC, human neutrophil collagenase; HS-(CH_2-*R*-Leu) = $HSCH_2(R)CH(CH_2CH(CH_3)_2)CO_2H$; analogous abbreviations are used for the *S* enantiomer and the racemic (*RS*) mixture; HO_2S- and HO_3S- designate the corresponding sulfinic acid and sulfonic acid derivatives, respectively; Bz-(Gly-S)-(CH_2-*R*-Leu) = $PhCONHCH_2SCH_2(R)CH(CH_2CH(CH_3)_2)CO_2H$; (GlySO) and (Gly-$SO_2$) designate the corresponding sulfoxide and sulfone derivatives, respectively.

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an appropriately placed metal coordinating group (8,9). Accordingly, vertebrate collagenase inhibitors containing sulfhydryl (10-16), hydroxamate (11,17,18), phosphoramidate (19) and phosphonamidate (20,21) moieties have already been developed. For a variety of reasons, some chemical and some pharmacological, it is desirable to develop alternate substrate analogs with novel functional groups. In this study, we have prepared a series of sulfur-based substrate analog inhibitors of the two human collagenases HFC and HNC. The changes in inhibitor potency with variation of the functional group provides insight into the enzyme-inhibitor interactions that are important for optimal inhibitor design for these collagenases.

MATERIALS AND METHODS

Synthesis of Inhibitors: Enantiomerically pure Leu-analog mercaptans HS-(CH_2-*R*-Leu) and HS-(CH_2-*S*-Leu) were unambiguously synthesized as their S-benzyl derivatives by adaptation of the Evans asymmetric synthesis procedure (22), utilizing (1*S*,2*R*)-(+)-norephedrine and (1*R*,2*S*)-(-)-norephedrine (Aldrich Chemical Co., Inc.), respectively, as chiral auxiliaries. The S-benzyl derivative of HS-(CH_2-*S*-Leu) prepared by this procedure exhibited $[\alpha]_D$-25.0° (c 2.04, abs. EtOH); its optical purity was estimated to be 90-95% by subsequent conversion (*vide infra*) to Bz-(Gly-S)-(CH_2-*S*-Leu)-Ala-NH_2 and comparison of its ^{13}C NMR spectrum with that of Bz-(Gly-S)-(CH_2-*RS*-Leu)-Ala-NH_2. The Leu-analog mercaptans were coupled as their S-benzyl or S-acetyl derivatives to the required amino acid or dipeptide amides using the dicyclohexylcarbodiimide/1-hydroxy -benzotriazole method, and then were deblocked by Na/NH_3 reduction (22) or alkaline hydrolysis to afford the mercaptan inhibitors (**1-7**, Table 1). Representative physical data: HS-(CH_2-*R*-Leu)-Phe-Ala-NH_2 (**2**), mp 174-176°C; ^1H NMR ($CDCl_3$, 300 MHz) δ 4.60-4.67 (m, 1H, Phe α-H), 4.51 (p, J=7 Hz, 1H Ala α-H), 1.37 (d, J=8 Hz, 3H, Ala Me), 0.74 (d, J=7 Hz, 3H, Leu Me), 0.64 (d, J=6 Hz, 3H Leu Me); HS-(CH_2-*S*-Leu)-Phe-Ala-NH_2 (**3**), mp 217-219 °C; ^1H NMR ($CDCl_3$, 300 MHz) δ 4.71 (q, J=7 Hz, 1H, Phe α-H), 4.44 (p, J=8 Hz, 1H, Ala α-H), 1.34 (d, J=7 Hz, 3H, Ala Me), 0.87 (d, J=7 Hz, 3H, Leu Me), 0.83 (d, J=7 Hz, 3H, Leu Me).

The disulfide (**8**) was prepared by base-catalyzed hydrolysis of the S-acetyl derivative of **7** in the presence of air. The sulfide derivatives (**12, 13**) were prepared by reaction of the mercaptans with N-(hydroxymethyl)benzamide in trifluoroacetic acid (23). Oxidation of the sulfides with *m*-chloroperbenzoic acid (24) furnished the sulfoxide (**14-17**) and sulfone (**18,19**) functionalities. The diastereomeric pairs of sulfoxides (**14-15, 16-17**) thus generated were separated by preparative thin-layer chromatography on silica gel using $CHCl_3$-EtOAc. Despite a recent suggestion to the contrary (25), the sulfoxide inhibitors were shown by ^1H NMR to be stable in protic media at 40°C indefinitely and at 50°C for at least six hrs. Base-induced elimination of the sulfones provided the corresponding sulfinic acids (**10,11**). The sulfonic acid (**9**) was prepared by direct oxidation of the S-acetyl derivative of the corresponding mercaptan with peracetic acid (26). The inhibitor structures were confirmed by ^1H NMR at 300 MHz, as well as by ^{13}C NMR, mass spectroscopy and combustion analysis as appropriate.

Enzymatic Assays and Inhibition Measurements: HNC was isolated from human buffy coats in an active 58 kDa form (27). HFC was isolated from fibroblast cultures as the 53 kDa zymogen (1). It was activated by incubation with 100 μg/ml trypsin for 15 min at 23°C and the trypsin inactivated with a 4-fold excess of soybean trypsin inhibitor prior to all assays. Collagenolytic activity was measured using rat type I [^3H]collagen as substrate at a concentration of 0.33 μM at 30°C as described elsewhere (28). Assays were carried out in 50 mM Tricine, 0.2 M NaCl, 10 mM $CaCl_2$, pH 7.5 containing 0.05% Brij-35 and 50 μM

$ZnCl_2$. Inhibitors were dissolved in either methanol or dimethylsulfoxide and diluted into the assays so that the final concentration of organic solvent was less than 5% v/v. The inhibition curves were corrected for the slight (< 20%) lowering of activity by these solvents. The concentrations of all stock solutions of sulfhydryl inhibitors were determined by reaction with Ellman's reagent (29) and found to agree to within 10% with the concentration prepared by weight, indicating that they were not oxidized. The state of the sulfhydryl group of **1**-**7** under assay conditions was also investigated and found to remain >80% reduced over the 4 hr time span of the assay. The inhibition results are expressed as the inhibitor concentration that produces 50% inhibition (IC_{50}) at the single substrate concentration used.

RESULTS AND DISCUSSION

Substrate analogs consisting of the P'_n part of the substrate (nomenclature of Schechter and Berger (30)) with the scissile peptide bond replaced by a $HS\text{-}CH_2$ group are known to be potent inhibitors of metalloproteinases (8,9,31,32). The sulfhydryl group is believed to interact with the active site zinc atom of the enzyme, a view that has been confirmed experimentally for certain complexes (9,33). Sulfhydryl compounds with subsite P'_1 occupied by Leu, P'_2 by Ala, Phe or Trp, and P'_3 by Gly or Ala have been shown to be good inhibitors of tadpole (12), rabbit corneal (10) and pig synovial (13,14) collagenases. The potency of these inhibitors is markedly dependent on the stereochemistry at the asymmetric carbon to which the Leu side chain is bonded, but the absolute configuration of the more potent diastereomer had not been assigned earlier (13). Thus, as a starting point for these studies, stereospecific synthesis has been used to prepare both the R-Leu and S-Leu diastereomers of each of three substrate analogs (**2**-**7**) and to quantitate their inhibition toward the more interesting human collagenases.

The inhibition results for HNC and HFC are summarized in Table 1. Compound **1** is a diastereomeric mixture of mercaptan analogs whose amino acid sequence mimics that found for the α2(I) chain at the collagenase cleavage site of chick type I collagen (34). Submicromolar IC_{50} values are observed toward both human collagenases. When the P'_2 amino acid is changed to Trp or Phe, and the P'_3 residue is changed to Ala or the $NH(CH_3)$ group, significantly lower IC_{50} values are observed for one of the two possible diastereomers (**2**-**7**). In contrast to previous studies (10,13), we find little (<20%) decrease in mercaptan inhibitor potency after incubation with the enzymes for 4 hr. This suggests that autooxidation of the mercaptan to the disulfide is not occurring under the assay conditions (compare **6** and **8**). Since the IC_{50} values reported for the disulfide (**8**) could be accounted for by less than 0.1% contamination with the parent mercaptan (**6**), these values must be considered upper limits only.

By virtue of the asymmetric synthesis approach used here, the stereochemistry of the more potent diastereomer in each of the mercaptan inhibitor pairs can now be assigned to that of the $HS\text{-}(CH_2\text{-}$*R*-Leu)-derivative. This stereochemistry corresponds rather surprisingly to the *unnatural* substrate (D-Leu) absolute configuration. There have been few reports of the assignment of stereochemistry at the P'_1 residue of analogous synthetic

Table 1. Inhibition of HNC and HFC by Sulfur-Based Substrate Analogs

NO.	INHIBITOR P_1 P'_1 P'_2 P'_3	IC_{50} (μM) HNC	HFC
1	HS–(CH_2-*RS*-Leu)-Ala-Gly-NH_2	0.53	0.89
2	HS–(CH_2-*R*-Leu)-Phe-Ala-NH_2	0.042	0.056
3	HS–(CH_2-*S*-Leu)-Phe-Ala-NH_2	0.36	0.45
4	HS–(CH_2-*R*-Leu)-Phe-NHMe	0.056	0.072
5	HS–(CH_2-*S*-Leu)-Phe-NHMe	0.24	0.45
6	HS–(CH_2-*R*-Leu)-Trp-Ala-NH_2	0.048	0.079
7	HS–(CH_2-*S*-Leu)-Trp-Ala-NH_2	0.40	1.3
8	[S–(CH_2-*R*-Leu)-Trp-Ala-NH_2]$_2$	≥79	≥130
9	HO_3S–(CH_2-*RS*-Leu)-Ala-Gly-NH_2	1100	1400
10	HO_2S–(CH_2-*R*-Leu)-Phe-Ala-NH_2	710	1100
11	HO_2S–(CH_2-*S*-Leu)-Phe-Ala-NH_2	79	220
12	Bz-(Gly-S)–(CH_2-*R*-Leu)-Phe-Ala-NH_2	110	210
13	Bz-(Gly-S)–(CH_2-*S*-Leu)-Phe-Ala-NH_2	25	36
14	Bz-(Gly-SO)–(CH_2-*R*-Leu)-Phe-Ala-NH_2 [a,c]	1100	1500
15	Bz-(Gly-SO)–(CH_2-*R*-Leu)-Phe-Ala-NH_2 [b,c]	560	79
16	Bz-(Gly-SO)–(CH_2-*S*-Leu)-Phe-Ala-NH_2 [a,c]	130	240
17	Bz-(Gly-SO)–(CH_2-*S*-Leu)-Phe-Ala-NH_2 [b,c]	580	710
18	Bz-(Gly-SO_2)–(CH_2-*R*-Leu)-Phe-Ala-NH_2	360	500
19	Bz-(Gly-SO_2)–(CH_2-*S*-Leu)-Phe-Ala-NH_2	140	350

[a] Unknown configuration at sulfur; higher R_f diastereomer (silica gel, $CHCl_3$-EtOAc).
[b] Unknown configuration at sulfur; lower R_f diastereomer (silica gel, $CHCl_3$-EtOAc).
[c] Slow-binding.

collagenase inhibitors. In the case of tripeptide analogs containing the hydroxamate functionality, high steroselectivity for inhibition was observed with the preferred inhibitor stereochemistry being that of the natural (L-Leu) absolute configuration (11). A sulfhydryl substrate analog in which the scissile bond was replaced by HS-CH_2-CH_2 showed only slight stereoselectivity, with the natural (L-Leu) analog being about twice as potent as the other diastereomer (11). The approximately 10-fold selectivity for the unnatural diastereomer in the **4**-**5** and **6**-**7** pairs is therefore unprecedented.

A new series of substrate-analog inhibitors has been synthesized in which the HS group of mercaptans **1**, **2** or **3** has been replaced by HO_3S to give the corresponding sulfonate, by HO_2S to give the sulfinate, by Bz-Gly-S to give the sulfide, by Bz-Gly-SO to give the sulfoxide, and by Bz-Gly-SO_2 to give the sulfone. The rationale for investigating these new compounds derives from several considerations. First, the bonding geometry to the sulfur atom in these derivatives is approximately tetrahedral. Since the carbonyl carbon that it replaces is thought to become tetrahedral in the transition state for peptide bond hydrolysis, the resultant derivatives could be transition-state analogs. Second, these new compounds offer potential sites for electrostatic interaction with the active site zinc atom and/or hydrogen

bonding to nearby protein residues. Last, the sulfides, sulfoxides and sulfones allow the peptide portion to be extended to include the P_n side of the substrate and could increase inhibitor potency through additional interactions with S_n sites on the enzyme (30). To the best of our knowledge, no metalloproteinase inhibitors of these types have been reported previously.

As can been seen from Table 1, all of the compounds containing these new sulfur-based functionalities are less potent collagenase inhibitors than are the corresponding mercaptans. The best inhibitor among these new compounds is **13** which exhibits IC_{50} values of 25 and 36 μM toward HNC and HFC, respectively. Only the sulfoxides exhibited slow-binding behavior with our assays and 16 hr incubations of enzyme and inhibitor were carried out prior to measuring the IC_{50} values. For the sulfinates (**10**,**11**), sulfides (**12**,**13**) and sulfones (**18**,**19**), the *S*-Leu are better inhibitors than the *R*-Leu isomers. The sulfoxides (**14**-**17**) have a new asymmetric center at the sulfur atom leading to an additional pair of diastereomers. The less polar (higher R_f) of the S-Leu diastereomers (**16**) is the best inhibitor of HNC, while the more polar of the R-Leu diastereomers (**15**) is the best inhibitor of HFC. These observations suggest different binding modes than those operative with the mercaptans. The sulfur atom is tetrahedral in the sulfonate, sulfinate, sulfoxide and sulfone functionalities and each can potentially offer the active site zinc atom an oxygen atom that is *in register* with the carbonyl oxygen of the substrate scissile peptide bond, making these compounds potential transition-state analog inhibitors. A range of IC_{50} values was clearly expected, since the metal-coordinating ability of these functionalities varies considerably. It was, however, very surprising that the sulfide **13** displays the best inhibition of any of these tetrapeptide analogs. The sulfonate and sulfinate functionalities prove to be ineffective despite having charged in-register oxygens available for binding to the zinc atom, as well as for hydrogen bonding to catalytic residues in the active site.

This unexpected variation in inhibitory potency with the different amide surrogate functional groups and surprizing variation in stereochemical preferences (Table 1) indicate that there is no simple rationale for the observed trends. While no structures of any mammalian collagenase-inhibitor complexes have been elucidated to date, the interactions between the metalloproteinase thermolysin and a number of inhibitors containing different functional groups have been determined crystallographically (35). Depending upon the inhibitor, each resulting complex has been proposed to mimic that of either a substrate, reaction intermediate, transition state or product bound to the enzyme. As a result, there is considerable diversity with respect to the interactions of the different amide surrogate functional groups with the enzyme. The two types of interactions that are common to all of these complexes, however, are coordination of one or more atoms of the functional group to the zinc atom and hydrogen bonding interactions with nearby protein residues. In interpreting the potencies of the inhibitors synthesized here for the human collagenases, similar enzyme-inhibitor interactions can reasonably be assumed to be in effect. Thus, the

IC_{50} values reflect the sum of many potential interactions. Unfortunately, the importance of any one of them is difficult to evaluate without the aid of a crystal structure. In the case of the mercaptan inhibitors, the preference for the unnatural isomers in subsite P'_1 clearly indicates that these compounds are not binding to the enzymes in the same manner as the analogous substrates. It might simply be that the strong mercaptide-zinc interaction in these complexes dominates in determining the mode of binding with the result that the R-isomers bind more tightly. The functional groups in the other inhibitors are all less effective zinc ligands, accounting in part for their lower inhibitory potencies. Since the order of potency does not correlate only with the coordinating ability of the functional group, however (e.g. the best sulfoxide and best sulfone are equally potent for HNC), other factors such as hydrogen bonding to nearby catalytic residues are clearly important.

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