Phosphinic peptide analogues as potent inhibitors of *Corynebacterium rathayii* bacterial collagenase

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Pseudo-substrate analogues of collagenase from *Corynebacterium rathayii*, in which the scissile peptide bond is replaced by a phosphinic moiety, were synthesized and evaluated as inhibitors of this enzyme. The phosphinic tetrapeptide, Z-Phe-$\psi(PO_2CH_2)$-Gly-Pro-Nle (**1**), was found to be a potent inhibitor of collagenase with a K_i value of 8 nM. Increasing the length of the phosphinic-containing inhibitors from tetra- to hepta-peptide size further improves the potency of these compounds. The heptapeptide analogue, Z-Phe-Gly-Pro-Phe-$\psi(PO_2CH_2)$-Gly-Pro-Nle-OMe, with a K_i value of 0.6 nM, is the most potent inhibitor reported to date for bacterial collagenases. A comparison between the phosphinic analogue Z-Phe-$\psi(PO_2CH_2)$-Gly-Pro-Nle (**1**) and the phosphonamide peptide Z-Phe-$\psi(PO_2NH)$-Gly-Pro-Nle (**2**) shows that for bacterial collagenase the replacement of a CH_2 by an NH group results only in a modest increase in affinity from $K_i = 8$ nM for compound **1** to $K_i = 6$ nM for compound **2**. Most of the phosphorus-containing inhibitors of this series are slow- or slow–tight-binding inhibitors with second-order rate constants for association and dissociation varying respectively for the k_{on} values from 1×10^3 to 26×10^3 $M^{-1} \cdot s^{-1}$ and for the k_{off} values from 3×10^{-4} to 2×10^{-5} s^{-1}. Interestingly, the lower affinity of the molecule containing a D residue in the P_1 position of the inhibitor, compared with the molecule with an L residue in this position, is mainly the consequence of a lower rate constant for association of these D stereoisomers with the enzyme. This study demonstrates that phosphinic peptide analogues are potent inhibitors of a bacterial collagenase. The development of new phosphinic peptides should lead to the discovery of potent inhibitors of other zinc metalloproteases. Details of how the analogues were synthesized are given in Supplementary Publication SUP 50176 (14 pages), which has been deposited with the British Library Document Supply Centre, Boston Spa, Wetherby, W. Yorkshire LS23 7BQ, from whom copies can be obtained on the terms indicated in Biochem. J. (1994) **297**, 9.

INTRODUCTION

The number of proteases known to contain a zinc atom in their catalytic sites has rapidly increased during the last few years and will doubtless continue to grow [1,2]. Several of them have been identified as playing important physiological roles [3–5], and therefore considerable efforts are currently being made to regulate their functions using new potent and highly selective synthetic inhibitors. Although potent and selective inhibitors of a few well-known members of the zinc metalloprotease family (thermolysin, carboxypeptidase A, angiotensin-converting enzyme and neprilysin) are available [6], for most of the other members such inhibitors remain to be developed.

For several years, our laboratory has been involved in the development of inhibitors of bacterial collagenases, enzymes that also belong to the zinc metalloprotease family [7–10]. The most intensively studied collagenases have been those from *Clostridium histolyticum* which hydrolyse collagen, gelatin and peptides containing proline at the Xaa-Gly bond in Xaa-Gly-Pro-Yaa sequences [11,12]. Other microbial collagenases, including those from *Acromobacter iophagus* [13], *Bacillus cereus* [14], *Treponema denticola* [15] and *Corynebacterium rathayii* [16,10], have also been shown to degrade collagen, gelatin and the synthetic substrate furylacryloyl-Leu-Gly-Pro-Ala. Both the substrate and inhibitor specificity exhibited by these bacterial collagenases support the view of a conserved binding cleft among all of them.

The development of potent inhibitors of bacterial collagenases is an important goal as several studies have underlined their role in some human pathogenesis [14]. More specifically, collagenases produced by certain bacteria of the human flora are considered to be involved in the aetiology of periodontal disease [17]. More generally, the involvement of other bacterial zinc metalloproteases in human pathogenesis, such as those produced by *Serratia marcescens* and *Pseudomonas aeruginosa* (two micro-organisms considered to be major causative agents of many opportunistic infections), should reinforce the current interest in developing potent inhibitors of these bacterial zinc proteases [18–20].

One attractive approach to the development of potent inhibitors of zinc proteases involves synthesis of peptide substrate analogues in which the scissile peptide bond is replaced by the tetrahedral phosphonamide moiety (PO_2NH) [21–23]. In fact, several studies of these phosphonamide inhibitors have demonstrated the usefulness of this strategy and have convincingly supported the view that these molecules act as transition-state analogues of the equivalent peptide substrates [24,25]. In the case of bacterial collagenases, phosphonamide peptides with the general structure R-$\psi(PO_2NH)$-Gly-Xaa-Yaa (R being an aryl-alkyl group and Xaa and Yaa being amino acids) also proved to be potent inhibitors of these collagenases [9]. However, it should be stressed that a major limitation to the use of phosphonamide derivatives is the chemical instability of the phosphonamide

Convention used: Ψ indicates that the peptide bond has been modified, and the formula of the group that has replaced this peptide bond is in parentheses.

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bond. The P-N bond is very easily hydrolysed in certain sequences, even at neutral pH, and this occurs more readily when the size of the N-terminal peptide fragment of the phosphonamide bond is increased [26,27]. This disadvantage considerably limits the usefulness of the phosphonamide bond for devising zinc endoprotease inhibitors, particularly in cases where, for potency or selectivity reasons, both the P_n and $P_{n'}$ positions of the inhibitors need to be involved.

These considerations have led us to develop new pseudo-substrate inhibitors of bacterial collagenase that are more stable and longer than the phosphonamide peptide inhibitors previously synthesized. In the present paper, we report a study of the potency and kinetic properties of a new series of phosphinic-containing peptides capable of interacting with the S_4–$S_{3'}$ subsites of the active site of *Corynebacterium rathayii* collagenase.

EXPERIMENTAL

Materials

Collagenase from *C. rathayii* [16] was purified from a crude extract by a two-step procedure. This material (1 mg/ml) was loaded on to a gel-filtration column (TSK SW3000; Tosho) equilibrated with 50 mM Tricine/NaOH buffer, pH 7, containing 10 mM $CaCl_2$ and 50 mM NaCl. Further purification was achieved by f.p.l.c. on an anion-exchange column (Mono Q; Pharmacia). The enzyme was eluted by applying an NaCl gradient (0.05–1 M). The major peak, containing activity against the furylacryloyl-Leu-Gly-Pro-Ala substrate, when analysed by SDS/PAGE, shows a single band in the 81 kDa molecular-mass range.

Assay of collagenase

Unless otherwise noted, all assays were performed under the following buffer conditions: 25 °C, pH 7.5, 50 mM Tricine/NaOH, containing 10 mM $CaCl_2$, 1 M NaCl and 1.25 mg/ml 1-*O*-octyl-β-D-glucopyranoside. The presence of both 1 M NaCl and the 1-*O*-octyl-β-D-glucopyranoside in the buffer resulted in substantial stabilization of collagenase activity without affecting the kinetic properties of the enzyme. Under these conditions, 0.1–1 nM samples of collagenase retain the same activity for 4 days at 25 °C. Collagenase activity was assayed using the synthetic substrate furylacryloyl-Leu-Gly-Pro-Ala, as described by Van Wart and Steinbrink [28]. Enzyme concentration was determined spectrophotometrically using a molar absorption coefficient of 1.5×10^5 $M^{-1} \cdot cm^{-1}$ at 280 nm.

Kinetic assays

Reaction progress was measured spectrophotometrically by monitoring the release of furylacryloyl-Leu at 330 nm during hydrolysis of furylacryloyl-Leu-Gly-Pro-Ala. In a typical experiment, a cuvette containing 2 ml of buffer and 20 μl each of solutions of substrate and inhibitor was brought to thermal equilibrium (5–10 min) in a jacketed holder in the cell compartment of a Cary-219 spectrophotometer. The temperature was maintained by water circulating from a Haake P2 bath. Injection of 5–15 μl of enzyme initiated the reaction. Absorbance was continuously measured, digitized and stored in a Macintosh FX computer, equipped with a MacADIOS II Jr data-acquisition system (GW Instruments). As an interface between the acquisition system and the computer the LABTECH NOTEBOOK software was used (Laboratory Technologies Corp.). Progress curves were composed of 500–1000 (absorbance, time) pairs.

Progress-curve analysis

Progress curves for the slow-binding inhibitors of collagenase were analysed using the integrated expression described by Morrison and Walsh [29].

$$P = v_s t + (v_0 - v_s)[1 - \exp(-k_{app.} t)]/k_{app.} \quad (1)$$

where P is product concentration, v_0 is the reaction velocity at $t = 0$, v_s is the final steady-state velocity and $k_{app.}$ is the observed first-order rate constant for the approach to steady-state.

To investigate the kinetic mechanism of inhibition of collagenase by the present series of inhibitors, several progress curves at different inhibitor and substrate concentrations were recorded, and successfully fitted to eqn. (1) by non-linear least-squares analysis. These procedures provide values for the three empirical parameters v_0, v_s and $k_{app.}$

Determination of K_i values

In some cases, slow-binding inhibitors impose long equilibrium times so that the equilibrium cannot be observed before complete depletion of the substrate. In these cases, measurements were made by equilibrating increasing concentrations of inhibitor in 2 ml of solution containing 0.1–1 nM enzyme overnight, and then initiating the reaction by adding 10 μl of substrate to determine the residual free enzyme concentration. Owing to the low dissociation rates for most of the inhibitors (see below), it was possible to neglect the effect of the addition of a competitive substrate on the equilibrium position. Determination of the equilibrium position makes it possible to calculate the K_i value using eqn. (2):

$$[EI] = 1/2\{(I_t + E_t + K_i) - [(I_t + E_t + K_i)^2 - 4E_t I_t]^{1/2}\} \quad (2)$$

which takes into account mutual depletion of enzyme (E) and inhibitor (I) [30,31].

Reversal of inhibition and determination of k_{off}

The first-order rate constant for dissociation of the enzyme–inhibitor complex was determined as follows: 0.5 ml of a solution of 1 μM collagenase and 10 μM inhibitor was incubated at room temperature for 20 min. This solution was then cooled to 4 °C and loaded on a HR 10/10 fast desalting column (Pharmacia) that had been equilibrated at 4 °C with 50 mM Tricine/NaOH buffer, pH 7.5, containing 10 mM $CaCl_2$. The column was eluted at 4 °C with this buffer, and the purified EI complex fraction was pooled and then rapidly diluted with the pH 7.5 buffer composed of 50 mM Tricine/NaOH, 10 mM $CaCl_2$, 1 M NaCl and 1.25 mg/ml 1-*O*-octyl-β-D-glucopyranoside to produce a 1 nM stock solution of the EI complex. This solution was kept at 25 °C and 2 ml samples were assayed periodically for return of the enzyme activity by adding 5 μl of a 12 mM stock solution of the furylacryloyl-Leu-Gly-Pro-Ala substrate in dimethylformamide.

When the inhibitor levels required to produce complete progress curves that achieved steady-state velocities before appreciable substrate depletion were in the range of the K_i value, the k_{off} value was determined by plotting $k_{app.}$ values obtained at different inhibitor concentration against the inhibitor concentration using eqn. (3):

$$k_{app.} = k_{off} + k_{on}(I)/(1 + S/K_m) \quad (3)$$

This equation assumes that EI complex-formation involved a

single step (see below in the text) [29]. All values reported for K_i and k_{on} were reproducible within ±5% and for k_{off} within ±12%.

Inhibitor synthesis

Details of how the analogues were synthesized are given in Supplementary Publication SUP 50176 (14 pages), which has been deposited with the British Library Document Supply Centre, Boston Spa, Wetherby, W. Yorkshire LS23 7BQ, from whom copies can be obtained on the terms indicated in Biochem. J. (1994) **297**, 9.

Table 1 Inhibition constants for binding of phosphinic and phosphonamide peptides to *C. rathayii* collagenase

Values for K_i were determined as described in the text. Assays were carried out in 50 mM Tricine buffer, pH 7.5, containing 10 mM $CaCl_2$, 1 M NaCl and 4.3 mM 1-*O*-octyl-β-D-glucopyranoside, 25 °C. Φ, phenyl.

Compounds (P_4-P_3-P_2-**(D,L)P_1**-ψ(PO_2X)-P_1'-P_2'-P_3')	K_i (nM) L isomer	K_i (nM) D isomer
1 Z-**Phe**-ψ(PO_2CH_2)-Gly-Pro-Nle	8	140
2 Z-**Phe**-ψ(PO_2NH)-Gly-Pro-Nle	6	90
3 Z-Pro-**Phe**-ψ(PO_2CH_2)-Gly-Pro-Nle	35	280
4 Z-Gly-Pro-**Phe**-ψ(PO_2CH_2)-Gly-Pro-Nle	1.3	32
5 Z-Gly-Pro-**Phe**-ψ(PO_2CH_2)-Gly-Pro-Nle-OMe	1.1	35
6 Ac-Gly-Pro-**Phe**-ψ(PO_2CH_2)-Gly-Pro-Nle-OMe	1.2	39
7 Z-Phe-Gly-Pro-**Phe**-ψ(PO_2CH_2)-Gly-Pro-Nle-OMe	0.6	25
8 Z-Gly-Pro-**(D,L)Phe**-ψ(PO_2CH_2)-Gly-Pro-OMe	1000	
9 Z-Gly-Pro-**(D,L)Phe**-ψ(PO_2CH_2)-Gly-OEt	12000	
10 Φ-CH_2-CH_2-ψ(PO_2NH)-Gly-Pro-Nle	50	

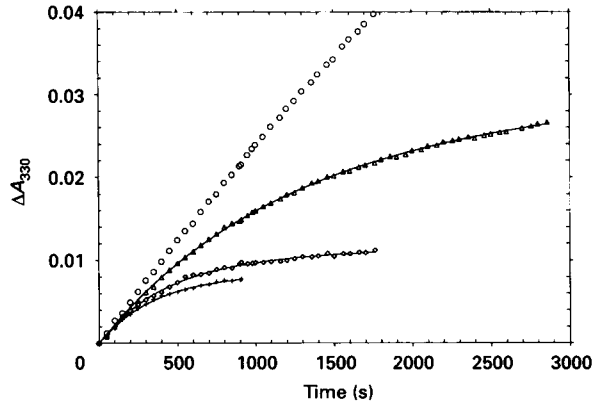

Figure 1 Time dependence of bacterial collagenase inhibition

Progress curves are shown for the slow-binding inhibition of 0.8 nM collagenase by Z-Phe-ψ(PO_2CH_2)-Gly-Pro-Nle in the presence of 60 μM furylacryloyl-Leu-Gly-Pro-Ala. Z-Phe-ψ(PO_2CH_2)-Gly-Pro-Nle concentrations were 0 (○), 0.1 (△), 0.3 (◇) or 0.5 (+) μM. Data points were collected every second, but, for clarity, only every fifty points are displayed. The solid lines through the points represent the best fit for each progress curve to eqn. (1). From these fits, a value of $k_{app.}$ was obtained for each inhibitor concentration.

RESULTS

Potency of the inhibitors

Synthesis of a pseudo-tetrapeptide substrate of *C. rathayii* collagenase containing a phosphinic bond in a suitable position produces a potent inhibitor of this zinc protease (compound **1**; Table 1). Furthermore, the parent phosphonamide peptide (compound **2**) is only slightly more potent than the phosphinic analogue (compound **1**). Elongation of the inhibitor on the N-terminal side of the phosphinic bond by a single residue decreases the affinity (compound **3**), whereas the addition of another residue (Z-Gly, compound **4**) produces a much more potent inhibitor. The K_i values of compounds **5** and **6** compared with that of compound **4**, show that the presence of either a free C-terminal carboxylate group or an N-terminal benzyloxycarbonyl group in the hexapeptide inhibitor has no influence on the activity. Nevertheless, further elongation of the N-terminal portion of the inhibitor with a hydrophobic residue (compound **7**) leads to further improvement in potency. In contrast with the above results, deletion of the Nle residue from the C-terminal side has a dramatic effect on activity (compound **8**). A further affinity decrease is observed after removal of the proline residue (compound **9**). Significantly, some of the phosphinic peptide inhibitors developed in this study are more potent than the arylalkylphosphonamide peptides previously reported [9] (see for example compound **10**).

Inhibition kinetics

In the absence of inhibitor, the steady-state rate of substrate hydrolysis by collagenase is reached instantaneously and remains nearly constant over the absorbance range monitored (Figure 1). In the presence of most phosphorus peptide inhibitors of the present series, there is a time-dependent decrease in the steady-state rate, which is a function of inhibitor concentration (Figure 1). The reaction-progress curves, as shown in Figure 1, were fitted to eqn. (1) by non-linear regression. The agreement between predicted and observed curves is satisfactory, indicating that this equation provides an adequate description of the inhibition. From these fits, $k_{app.}$, the apparent first-order rate constant for the approach to steady-state, can be obtained for different inhibitor concentrations.

The slow-binding behaviour described by eqn. (1) is consistent with different mechanisms [32]. A single-step process is represented by mechanism A:

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI$$

for which:

$$k_{app.} = k_2 + [k_1[I]/(1 + S/K_m)]$$

Mechanisms B involves a two-step process:

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EI^*$$

for which:

$$k_{app.} = k_4 + k_3[(I/K_i)/(1 + S/K_m + I/K_i)]$$

Each mechanism is distinguished, in theory, by the nature of the dependence of the first-order association rate constant on inhibitor concentration. The plot of $k_{app.}$ versus [I] (Figure 2) is linear over the range of inhibitor concentrations studied (0.01–0.5 μM), suggesting that inhibition of collagenase by compound **1** occurs by a single-step mechanism (mechanism A) [32].

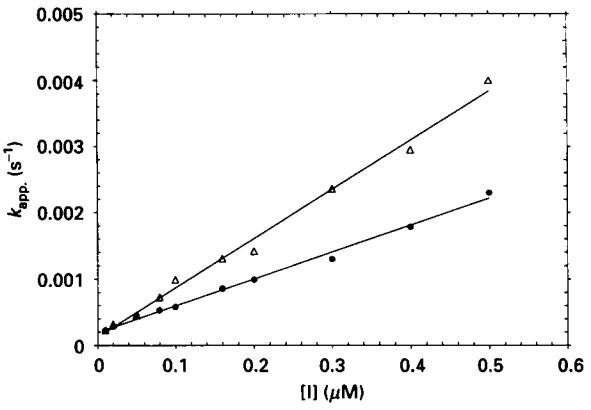

Figure 2 Determination of the k_{on} rate for the binding of Z-Phe-ψ(PO_2CH_2)-Gly-Pro-Nle to bacterial collagenase

Rate constants, $k_{app.}$, were determined at two substrate concentrations: 60 μM (2 K_m; △) and 150 μM (5 K_m; ●). Each point is the average of three experiments. These plots establish that the inhibition is competitive. The fits to eqn. (3) give the rate constant for association of Z-Phe-ψ(PO_2CH_2)-Gly-Pro-Nle: $k_{on} = 2.1 \times 10^4$ $M^{-1} \cdot s^{-1}$.

Table 2 Structural dependence of the association and dissociation rate constants of phosphonamide and phosphinic peptide inhibitors

The k_{on} and k_{off} values were determined from the progress curves using eqn. (3), as shown in Figures 1 and 2. The k_{off} values were determined from dissociation of the purified EI complex, followed by recovery of the activity. Conditions were as described in Table 1.

Compounds [P_3-P_2-(D,L)P_1-ψ(PO_2X)-$P_{1'}$-$P_{2'}$-$P_{3'}$]		k_{on} ($M^{-1} \cdot s^{-1}$)	k_{off} (s^{-1})
1 Z-**Phe**-ψ(PO_2CH_2)-Gly-Pro-Nle	(L)	21×10^3	2×10^{-4}
	(D)	1.8×10^3	3×10^{-4}
2 Z-**Phe**-ψ(PO_2NH)-Gly-Pro-Nle	(L)	20×10^3	1.5×10^{-4}
	(D)	1.5×10^3	1.8×10^{-4}
4 Z-Gly-Pro-**Phe**-ψ(PO_2CH_2)-Gly-Pro-Nle	(L)	26×10^3	2×10^{-5}
	(D)	1.8×10^3	4×10^{-5}

The decrease in rate constant with increasing substrate concentration indicates that compound **1** is a competitive inhibitor. The solid lines in Figure 2, predicted by mechanism A, correspond to a second-order association rate constant, k_{on}, of 2.1×10^4 $M^{-1} \cdot s^{-1}$ for compound **1**.

Similar slow-binding behaviour was observed for compounds **2** to **9** (results not shown). Second-order association rate constants of compounds **2** and **4** with collagenase, determined using the procedure outlined for compound **1**, are collected in Table 2. A salient feature of these data is that the rate of formation of complex EI is influenced by the stereochemistry of the side chain of the residue in the P_1 position of the inhibitor. For these inhibitors, the k_{on} values for the D compound is at least 10-fold lower than that for the L compound.

The slow- or slow–tight-binding inhibition observed with the present inhibitors is not only due to the low on rate for EI formation, but results also from rate constants measured for dissociation of the EI complex. Such rate constants correspond to an average residence time in the enzyme active site of 296 min for compound **4**, a very slow process compared with classic, even potent, inhibitors. From the data reported in Table 2, it appears that the difference in activity observed between the L and D stereoisomers in mainly due to the lower association rate displayed by the D isomers, compared with the L isomers.

Values of K_i, as calculated from $K_i = k_{off}/k_{on}$, are in close agreement with those determined on the basis of percentage of activity observed at equilibrium.

DISCUSSION

Synthesis of phosphorus-containing analogues of peptide substrates appears to be a very attractive approach to the development of zinc metalloprotease inhibitors. In fact, this strategy makes possible the synthesis of inhibitors that could contain amino acid side chains in the P_n–$P_{n'}$ positions capable of interacting with the corresponding S_n–$S_{n'}$ subsites of the enzyme active cleft. Therefore it should be suitable for developing specific inhibitors of zinc metalloproteases with a recognized extended active site, such as astacin [33,34], meprin A [35], mammalian collagenases [36], gelatinases [37] and stromelysin [38]. Furthermore, as pointed out by Lolis and Petsko [39], phosphorus-containing inhibitors of zinc metalloproteases, being good transition-state mimics, represent an unrivalled aid to the understanding of the source of enzyme catalytic power and specificity in crystallographic studies of enzyme complexes.

To develop such transition-state analogues, the use of the phosphonamide group as a surrogate for the scissile peptide bond appears at first sight to be the most suitable choice. However, as noted in the Introduction, the phosphonamide bond can be easily cleaved, depending not only on the pH of the medium, but also on the sequence and size of the phosphonamide peptide. Hanson et al. [26] reported that the Z-Ala-Ala-ψ(PO_2NH)-Phe phosphonamide peptide has a half-life of 20 min at pH 7.5, although this peptide is more stable at higher pH. A similar lability of the phosphonamide bond has also been observed for Z-Pro-Ala-Gly-ψ(PO_2NH)-$LeuNH_2$ [27].

To overcome this lability problem, the chemistry of peptides containing a phosphonate group has been developed [26,40]. However, we have previously shown that, for bacterial collagenases, the replacement of the phosphonamide bond by a phosphonate bond results in dramatic loss of potency [9]. The involvement of the NH group of the phosphonamide bond in hydrogen-bonding with some groups in the bacterial collagenase active site has therefore been postulated. Interestingly, despite this probable NH bond interaction, we have found in the present work that replacement of an NH by a CH_2 group has only a marginal effect on potency and thus provides another interesting alternative for overcoming the above lability problem. Such a result supports the prediction of Merz and Kollman [41] on the relative affinity of phosphonamide and phosphinic peptide inhibitors of thermolysin, based on free-energy-perturbation simulations. From their study, it has been argued that the loss of an NH active-site interaction, on going from a phosphonamide to a phosphinic group, should be balanced by the difference in solvation energy between the two groups. Since this theoretical study, experimental work validating these proposals has been carried out showing indeed that phosphonamide and phosphinic peptide inhibitors of thermolysin display almost the same potency [42]. On the basis of these results and our data, phosphinic instead of phosphonate peptides should be preferred for developing potent inhibitors of enzymes for which interactions between the NH group of the scissile peptide bond and the enzyme active site are presumed. It will be of interest to establish whether this proposal applies to other zinc metalloproteases. In

fact, to our knowledge comparison of the potencies of pseudopeptide containing amino acid residues on both sides of a phosphinic or a phosphonamide bond has been reported only in the case of the prototypic thermolysin protease [42,43].

The high potency displayed by the phosphinic compound **1** makes it possible to develop longer and more stable peptide inhibitors, capable of interacting with the S_4–$S_{3'}$ subsites of the collagenase active site. Compound **7**, with a K_i value of 0.6 nM, is the most potent inhibitor of bacterial collagenase reported to date. The moderate effect on activity associated with lengthening the N-terminal peptide chain sharply contrasts with the dramatic loss of potency observed with C-terminal truncation of the inhibitor (compounds **8** and **9**). These data underscore the critical role played by the C-terminal region of the inhibitor with respect to binding efficiency. Similar critical roles for the distal residues in either the C- or N-terminal portion of substrates and inhibitors on catalytic and binding efficiency have been reported for several proteases. Interestingly, Niedzwiecki et al. [38] reported that, for human stromelysin (a zinc protease), N-terminal truncation of the substrate results in a much greater reduction in catalytic efficiency than C-terminal truncation.

Under the experimental conditions used in this work, inhibition of bacterial collagenase by most of the inhibitors examined is consistent with a slow-binding mechanism as reviewed by Morrison and Walsh [29]. This inhibition behaviour is best described by mechanism A, a one-step process involving the slow binding of the inhibitor to the enzyme with no subsequent isomerization of the enzyme–inhibitor complex. However, it should be stressed that the two mechanisms are experimentally indistinguishable when the usable inhibitor concentration is one order of magnitude or more below the dissociation constant of the first enzyme–inhibitor complex in mechanism B [29]. The very low rates for both the association and dissociation steps of these inhibitors complicate the determination of both the K_i and k_{off} values. In fact, experimental conditions under which enzyme activity is stable over a long period of time are required in order to determine these constants[44]. Several examples of such slow binding have been reported for enzyme inhibitors, some of which are transition-state analogues [29,39]. Nevertheless, slow binding is by no means an absolute requirement for transition-state analogues. The exact origin of the slow-binding behaviour – conformational changes, water displacement – is still very controversial, and cannot be addressed further at this stage of the study [45]. However, the influence of stereochemistry in the P_1 position of the inhibitor on the k_{on} value is an interesting observation. In fact, as remarked by Kaplan et al. [44], it would be fruitful to have a better understanding of the different factors that determine the k_{on} value, in order to devise new inhibitors with faster association steps rather than very low off rates.

In conclusion, as compared with the series of arylalkylphosphonamide peptides previously reported [9], this new series of compounds contains more potent inhibitors of bacterial collagenase. The greater potency of the phosphinic compounds results mainly from additional interactions provided by amino acid residues in the P_4, P_3, P_2 and P_1 positions of these new inhibitors. Another significant difference between these two series of inhibitors concerns their kinetic behaviour. Whereas the previously developed inhibitors exhibit classic kinetic properties, the present compounds are slow–tight-binding inhibitors, characterized by very slow on and off rates. Further studies are required to explain these differences.

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