Cyclic Peptides with a Phosphinic Bond as Potent Inhibitors of a Zinc Bacterial Collagenase

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A series of cyclic peptides containing a phosphinic bond were synthesized and evaluated as inhibitors of a zinc bacterial collagenase from *Corynebacterium rathaii*. Among this series of pseudopeptides of different sizes of cycles, only two molecules **Ia** (cyclo[Gly-Pro-PheΨ(PO2CH2)-Gly-Pro-Ahx]) and **Va** (cyclo[βAla-Pro-PheΨ(PO2CH2)Gly-Pro-Ahx]) were found to be rather potent inhibitors of this protease, with K_i values of 120 and 90 nM, respectively. Besides the influence of the peptide ring size, this study suggests that both the stereochemical and the conformational properties of the pseudophenylalanine residue in these cyclic peptides may determine their potency. Interestingly, the kinetic analysis for the binding of the cyclic peptide inhibitors **Ia** and **Va** to the collagenase, as compared to a linear parent compound, reveals that the lower potency of the cyclic peptides is mostly the consequence of a lower rate constant for association to the enzyme. To our knowledge, this is the first report on cyclic phosphinic peptides and on their activities as inhibitors of a zinc protease.

Introduction

A number of bacteria have been shown to produce true collagenases.[1-7] Two main functions of these collagenases have been proposed: (1) a nutritional role, the hydrolyzed collagen or other proteins provide peptides that meet the nutritional requirements of these bacteria; (2) as a means for the invasion of the host tissue by these microorganisms. In this connection, the ability of certain bacteria present in the human flora to hydrolyze collagen has raised important considerations for human periotondal diseases.[5,8] Thus, there has been interest in the development of potent synthetic inhibitors of these proteases in order to prevent an extensive degradation of the collagen, but also to potentialy interfere with the bacterial growth.[9]

Several of these bacterial collagenases have been shown to hydrolyze collagen, gelatin, and peptides at the Xaa—Gly bond in an Xaa-Gly-Pro-Yaa sequence.[10,11] Both the substrate and inhibitor specificity exhibited by the collagenases from different microorganisms suggest the existence of a conserved binding cleft between all of these proteases.[4-6,12-14] For several years, our laboratory has been involved in the development of synthetic inhibitors of bacterial collagenases. Since these collagenases belong to the zinc protease family, several types of peptides containing, in a suitable position, a chemical group capable of interacting with the zinc atom present in the active site of these proteases were designed and synthesized as potential inhibitors of these enzymes.[12-15] More recently, peptide substrates containing a phosphinic bond, developed to mimic the substrate in the transition state, were proved to be very potent inhibitors of these collagenases.[16] From these studies, besides the importance of the zinc chelating group of these inhibitors, the essential requirements for a successful inhibition of the enzyme appear to be the following: (1) the inhibitor should contain at least four residues interacting, respectively, with S_1 through $S_{3'}$ subsites of the active site cleft, (2) the P_1 position should be occupied preferentialy by a bulky hydrophobic group, (3) a strict requirement in the $P_{1'}$ and $P_{2'}$ positions is observed, respectively, for glycine and proline, and (4) in the $P_{3'}$ position, either a linear basic or apolar side chain is preferred (Lys or Ahx). For example, the pseudotetrapeptide Z-PheΨ(PO2CH2)Gly-Pro-Ahx, which fulfills the above requirements, displays a K_i value of 8 nM when tested on the bacterial collagenase from *Corynebacterium rathaii*.[16]

In addition to these requirements for the primary structure of the inhibitor, on the basis of the crucial role played by the proline residue in the $P_{2'}$ position, a proline-induced bent conformation has been proposed to be a key element of the ligand binding process.[14,15] This latter proposal has led us to consider the design of cyclic peptides as potential inhibitors of this class of proteases. This approach should emphasize the conformational requirements of the inhibitor binding to the active site of bacterial collagenases, but also may represent an interesting issue for the control of the selectivity of these inhibitors. In fact, peptide inhibitors designed to block the bacterial collagenases were recently proved also to be potent inhibitors of the unrelated 24–15 and 24–16 mammalian zinc endopeptidases.[17] Furthermore, the discovery of active cyclic peptides is a crucial step for the development of rationally designed peptidomimetics.

In this study, the design and the potency of cyclic pseudopeptides containing a phosphinic bond as zinc chelating group are reported. The kinetic properties of the two more potent inhibitors of this series were also determined and are discussed.

Design

In the past decade many conformationally constrained cyclic analogues have been widely used in peptide structure–activity studies.[18-20] Most of these cyclic

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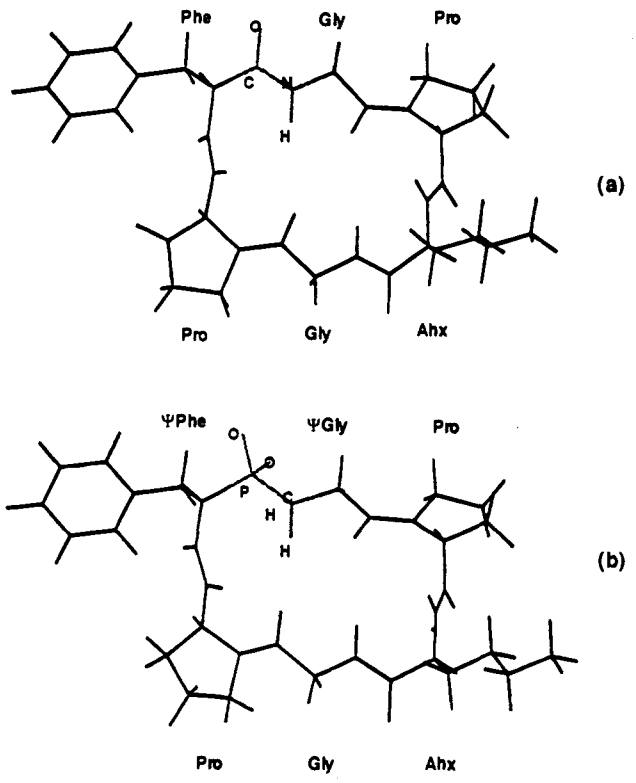

Figure 1. (a) Molecular model showing the cyclo[Gly-Pro-D-Phe-Gly-Pro-Ahx] molecule in a standard βII/βI turns conformation. (b) Molecular model for the cyclo[Gly-Pro-D-PheΨ-(PO_2CH_2)-Gly-Pro-Ahx]. The peptide bond in a was replaced by the phosphinic bond, and the resulting structure was energy minimized.

peptides were designed in order to interact with peptide hormone receptor, but very few against proteases.[21,22] An obvious reason for this is the fact that an extended conformation of the substrate or inhibitor peptides in the bound state has been in many cases observed in the proteases. However, for some proteases, including the bacterial collagenases, astacin[23] and meprin,[24] a bent conformation of the ligand has been proposed to be a key element of the proteases recognition. Therefore, for these proteases, cyclic peptides may represent interesting lead compounds.

The design of the cyclic peptides has very often taken advantage of the knowledge of the conformational properties of these cyclic molecules. Among these, from the conformational point of view, the most well studied have been the cyclic peptides containing five and six residues.[25,27] Extensive conformational analysis of these cyclic peptides has shown that these molecules adopt a stable "canonic" backbone conformation in solution. For the cyclic hexapeptides, this "canonic" backbone conformation is characterized by the presence of two β-turns connected by two residues in an extended conformation (Figure 1a), while for the cyclic pentapeptide this canonic structure corresponds to a β-turn fused with a γ-turn.

Other important contributions to this field have been the studies devoted to the development of the rules which operate to position a sequence of six or five residues on this framework; that is the phase problem. When a proline residue is present in the sequence of a cyclic hexapeptide, in a first approximation, these rules predict that the proline will occupy the (i + 1) position of the β-turn. This prediction has been in particular verified when the residue preceding the proline in the sequence does not contain a bulky side chain.[25]

The above rules have been used to design and predict the backbone structure of different cyclic analogues containing the sequence PheΨ(PO2CH2)Gly-Pro-Ahx, or a part of this sequence, in order to ensure the recognition of these cyclic peptides by the bacterial collagenases. The predicted backbone conformation of such a cyclic pseudopeptide is depicted in Figure 1b. For this hexapeptide to favor the presence of the canonic backbone conformation, the glycine–proline residues have been selected as a bridge segment to close the peptide ring. The two proline residues in this cyclic hexapeptide are predicted to occupy the (i + 1) position of the two β-turns, with the glycine residues at the junction positions. This structure has been devised from a cyclic hexapeptide in a standard βII/βI conformation (Figure 1a). An interesting feature of the model, in Figure 1b, is that the two oxygens of the hydroxyphosphinyl group should point toward the outside of the peptide macrocycle, a property which is a requisite to permit a subsequent interaction between these oxygens and the zinc atom of the enzyme active site.

In the same way, a β-turn + γ-turn structure can be predicted for the cyclic pseudopentapeptide (see Table 1).[25] For the heptapeptide, based on a study reported by Peishoff et al.,[28] it appears that a β-turn + β-bulge structure should be retained only if the C-terminal proline residue is pushed one place further in the sequence. Other analogues of the cyclic hexapeptide were designed in order to increase (Ser[1], ΔAla[1] analogues) or decrease (β-Ala[1]) the rigidity of the peptide macrocycle. In the same way, the use of a disulfide bridge to close the ring should lead to a more flexible structure.

Results and Discussion

Inhibitory Potency. Cyclic peptides are generally recognized as being resistant to proteolytic degradation.[29,30] However, the possibility that the observed inhibition in the present case is due to a linear product, resulting from a cleavage of the cyclic peptides by collagenase, can be envisaged. In fact, bacterial collagenase cleaves specifically the peptide bond preceding the glycine residue in a Xaa-Gly-Pro-Yaa sequence. Such a sequence is presented in Peptide **I** and **III** (Ahx-Gly-Pro-ΨPhe in **I** and Ala-Gly-Pro-ΨPhe in **III**). So, in order to rule out this possibility, for these cyclic peptides we have checked that their levels of inhibition, as well as their $k$on values, are not influenced, under our experimental conditions, by the concentration of the enzyme (e.g., by a catalytic process). Furthermore, for enzyme–inhibitor solutions yielding 50% of inhibition, we have also checked that the level of inhibition observed is the same for different times of enzyme–inhibitor preincubation (12, 24, 36, and 48 h, respectively). In addition to the above arguments, the data reported below also support an inhibition mechanism of the bacterial collagenase by these cyclic peptides, which is independent of an hydrolytic process.

The analysis of the binding constants (K_i values) of the cyclic phosphinic peptides containing a pseudophenylalanine residue with L or D stereochemistry in the P_1 position of the molecule shows that many of these cyclic peptides exhibit a moderate potency against the

bacterial collagenase, with the exception of the two compounds **Ia** and **Va**, which can be considered as rather potent inhibitors (K_i = 120 nM and K = 90 nM, respectively). However, it should be noted that the parent linear peptide (compound **VIIIa**) is more potent than these two cyclic peptides. The lower potency displayed by the cyclic peptides is probably not due to a lack of a free C-terminal carboxylate group in these molecules, since the esterification of the C-terminal group in the linear inhibitor have been shown to result only in a minor change of the potency.[16] Thus the conformation stabilized in these cyclic peptides probably does not correspond to the bioactive conformation recognized by the enzyme active site. Alternatively, or in addition to this, these cyclic molecules might encounter, during their binding to the active site, some steric hindrance which is not obviously easily overcome in the case of a constrained molecule.

In this respect, it is interesting to notice that the more constrained cyclic peptides (compounds **VI** and **VII**), as compared to compound **I**, are less potent, while the indroduction of one methylene in the ring, by replacing the glycine by a β-alanine, slightly improves the potency of the inhibitor (compound **V**). This positive effect on potency by increasing the ring flexibility might also explain the slightly better affinity of compound **IVa**, which contains a disulfide bridge, as compared to the potency of compounds **II** and **III**.

Another factor, which is interesting to consider in the analysis of structure–activity data, concerns the existence of a common binding mode for compounds with similar structures.[31] The different effect on the inhibitory potency of the stereochemistry of the residue in the P_1 position of the inhibitor, as observed between the cyclic peptides and the linear one, may emphasize that this view corresponds to an oversimplification in the present case. In fact, it can be seen from Table 1 that the modifications of the stereochemistry of the pseudophenylalanine residue in the linear peptide affects the affinity by a factor of 18, while the same modification in the cyclic analogues only modifies the potency by a factor less than 2, with the exception of compound **IV**. This result is somehow surprising, as our previous studies have shown that both the presence and the orientation of the phenylalanine side chain is a critical factor for ensuring a tight binding. Thus, the slight difference in the potency displayed by the L and D isomers of the cyclic peptides may indicate that the phenylalanine side chain plays only a marginal role in the binding efficiency of the L isomers, a factor which may explain the lower potency of the L cyclic peptides, as compared to the L linear one.

As mentioned above, in all these cyclic peptides, the phenylalaninephosphinic residue is presumed to occupy the position (i + 2) of a β-turn, with the hydroxyphosphinyl group pointing outside of the peptide macrocycle. It will be thus helpful to determine the 3D structure of these cyclic peptides in order to check the validity of this prediction. It is noteworthy that due to the presence of the hydroxyphosphinyl group, these cyclic peptides, which contain only apolar side chains, are rather soluble in water, and thus the 3D structure of these molecules could be determined by NMR spectroscopy in this solvent.

Table 1. Inhibition Constants for Binding of Cyclic Phosphinic Peptides to Bacterial Collagenase from *Corynebacterium rathayii*[a]

	Compounds P_3—P_2—(D,L)P1Ψ(PO_2CH_2) P_1—P_2'—P_3'		Ki μM
Ia	cyclo·[Gly-Pro·PheΨ(PO_2CH_2)Gly·Pro-Ahx]	(L)	0.12
Ib		(D)	0.18
IIa	cyclo·[Gly-Pro·PheΨ(PO_2CH_2)Gly·Pro]	(L)	6
IIb		(D)	9
IIIa	cyclo·[Gly-Pro·PheΨ(PO_2CH_2)Gly·Ala·Pro·Ala]	(L)	12
IIIb		(D)	20
IVa	Ac·Cys-Pro·PheΨ(PO_2CH_2)Gly-Pro-Cys·OMe (S—S)	(L)	3
IVb		(D)	45
Va	cyclo·[βAla·Pro·PheΨ(PO_2CH_2)Gly-Pro·Ahx]	(L)	0.09
VIa	cyclo·[Ser-Pro·PheΨ(PO_2CH_2)Gly·Pro·Ahx]	(L)	25
VIb		(D)	40
VIIa	cyclo·[ΔAla-Pro·PheΨ(PO_2CH_2)Gly-Pro·Ahx]	(L)	30
VIIIa	Z·PheΨ(PO_2CH_2)Gly·Pro·Ahx	(L)	0.008
VIIIb		(D)	0.14

[a] Values for K_i were determined as described in the text. Assays were carried out in 50 mM Tricine buffer, pH 7.5, 10 mM $CaCl_2$, 1 M NaCl, 4.3 mM octyl β-D-glucupyranoside, 25 °C.

Table 2. Association and Dissociation Rate Constants of Cyclic Phosphinic Inhibitors

	Compounds	k_{on} M^{-1}, sec^{-1}	k_{off}[a] sec^{-1}
VIIIa	Z·PheΨ(PO_2CH_2)Gly·Pro·Ahx	$20\ 10^3 \pm 2$	$1.6\ 10^{-4}$
Ia	cyclo·[Gly-Pro·PheΨ(PO_2CH_2)Gly·Pro·Ahx]	$1.6\ 10^3 \pm 0.2$	$2\ 10^{-4}$
Va	cyclo·[βAla·Pro·PheΨ(PO_2CH_2)Gly-Pro·Ahx]	$3.5\ 10^3 \pm 0.5$	$3.2\ 10^{-4}$

[a] The k_{off} values were determined from $k_{off} = K_i k_{on}$.

Kinetic Studies. Previous kinetic analysis of a series of linear phosphinic peptides, as inhibitors of bacterial collagenase, has shown that these molecules are slow binding inhibitors. The second-order rate constants ($k$on), as measured for the compound **Ia** and **Va**, indicate that the cyclic peptides are also very slow binding inhibitors (Table 2). Furthermore, the comparison of the $k$on values displayed by the cyclic peptides **Ia** and **Va** and the linear peptide (**VIIIa**) makes it possible to conclude that the lower potency of the cyclic molecules is mostly the consequence of a lower rate constant for association to the enzyme. This effect may, in part, result from an inadequate orientation of the phenylalanine side chain in these cyclic molecules. In fact, we have previously observed that the stereochemistry of the phenylalanine residue in the linear

peptide inhibitors influences their binding kinetics.[16] Besides the orientation of this side chain, its conformational constraint in these cyclic peptides is probably another factor which plays a role in the binding process. In this connection, the increase of the association rate constant observed for the compound **Va**, as compared to compound **Ia**, may be due to the existence of a direct link between the *k*on value and the rigidity of the inhibitor structure. This hypothesis correlates with the higher *k*on value measured in the linear peptide. Unfortunately, the 2-fold increase in the *k*on value noticed for the β-alanine compound does not lead to a 2-fold increase of the potency, because of the increase of the *k*off values (Table 2).

Conclusions

Two cyclic peptides containing a phosphinic bond and the required sequence to ensure their recognition by a bacterial collagenase were shown to be rather potent inhibitors of this zinc metalloprotease. These results support the previous proposal that a bent conformation of the ligands could be a key element of their recognition by the bacterial collagenase.[14,15] Furthermore, this study provides insights into the design of new cyclic inhibitors which may lead in the near future to the improvement of their potency. One possibility seems to increase the flexibility of the peptide macrocycle. Another interesting issue is to design a new aromatic phosphinic residue in order to restore a tight interaction between the side chain in the P_1 position of the inhibitor and the collagenase active site.

The development of very potent cyclic pseudopeptide inhibitors of bacterial collagenases represents an interesting challenge, not only because such molecules may lead to a rational design of peptidomimetics as inhibitors of these enzymes,[32,33] but also such a strategy might be extended to the design of other cyclic peptides, as potent and selective inhibitors of the mammalian endopeptidases 24−15 and 24−16, or as inhibitors of other zinc metalloproteases, a field in rapid expansion. The determination of the 3D structures of these cyclic pseudopeptides in aqueous solution are underway in our laboratory and will be reported.

Experimental Section

General. The convention used to described the peptide bond surrogates is that introduced by Spatola in 1983.[34] The Ψ letter code indicates that the peptide bond has been modified, followed in parentheses by the formula of the group that has replaced this peptide bond.

Melting points were taken on a hot stage and are uncorrected. Spots on thin-layer chromatograms were performed on silica gel plates (E. Merck silica gel 60 F-254), and components were visualized by the following methods: ninhydrin spray, exposure to hydrogen chloride vapor, followed by ninhydrin for protected and deprotected peptides, ultraviolet light absorbance, iodine vapor and charring after spraying with a solution of $(NH_4)HSO_4$. The solvent systems used for TLC development were: (1) 1-butanol−acetic acid−water (4:1:1), (2) chloroform−methanol−acetic acid (7:2:1), (3) 1-propanol−water (64:36), (4) 1-butanol−pyridine−0.1% acetic acid (5:3:11), (5) acetic acid−water−acetone−2-propanol (20:50:75:55).

Preparative HPLC purification was performed on a Gilson gradient system equipped with variable-wavelength detector. Compounds were detected at 254 and 230 nm. The following conditions were used: Vydac C18 (4.66 × 25 cm) column, mobile phase, A = 0.1% TFA, 10% acetonitrile in H2O, B = 0.1% TFA, 10% H_2O in acetonitrile, flow 8 mL/min, gradients: (1) T = 0 min, 0% B; T = 6 min, 20% B; T = 22 min, 35% B; T = 30 min, 100% B; (2) T = 0 min, 0% B; T = 22 min, 18% B; T = 30 min, 100% B. Analytical HPLC was performed with Vydac C18 (0.21 × 25 cm) column, flow rate 1 mL/min, using the same mobile phases and gradients as described above.

^1H and ^{31}P NMR spectra were recorded on the AMX 500 (^1H, 500 MHz; ^{31}P, 202 MHz) and WM 250 (^1H, 500 MHz; ^{31}P, 101 MHz) Bruker spectrometers. ^{31}P decoupled spectra were obtained on the AMX 500 instrument using a B-SV3 broadband modulator and a 5-mm $^1H/^{31}P$ dual probe. Broad-band or selective ^{31}P decoupling was achieved by choosing the proper pulse sequence and power for the ^{31}P in the transmitter channel while observing the proton in the reverse mode. All the free phosphorus peptides were dissolved in H_2O or D_2O (Commissariat à l'Energie Atomique).

^1H NMR chemical shifts are reported on δ scale (in ppm) relative to sodium 3-(trimethylsilyl)[2,2,3,3-$^2H_4$]propionate in H_2O or D_2O solutions; ^{31}P NMR chemical shifts are reported downfield from 85% H_3PO_4. Data are presented as chemical shift (multiplicity, number of protons, coupling constant in hertz).

Fast atom bombardment mass spectrometry (FAB-MS) was performed on a Nermay R3010 threefold quadrupole instrument by Dr. Virelizier (DCC/DPE/SPEA/SAIS in CE-Saclay 91191 Gif-sur-Yvette).

Chemistry Procedures. General Procedures: All the pseudopeptides described in this work were synthesized by liquid phase procedures. The C-terminal extension of the common fragment (-PheΨ{PO(OH)CH_2}Gly-) was achieved in all cases using the fragment condensation approach. The couplings were performed using the carbonyldiimidazole method without the protection of the hydroxyphosphinyl group as described by Grobelny et al.[35] and by Yiotakis et al.[16] The elongation of the N-terminal part of the pseudopeptides was achieved with either the fragment condensation (compounds **I−III**) or the stepwise extension (**IV−VII**), using preformed *p*-nitrophenyl ester of the suitable amino acid or dipeptide for the couplings. This strategy makes it possible to synthesize all the free hydroxyphosphinyl linear compounds with good yields[16] and to avoid the problems mentioned in the literature.[36−39] In the same way, in order to separate the activation step from the coupling one, the azide method[40] was chosen to carry out the cyclization in the presence of the free hydroxyphosphinyl group. For the synthesis of compound **VII**, the dehydroalanine was formed after the cyclization, and not before as generally reported.[41] This strategy was devised not only to isolate compound **VI** during the synthesis but also due to the the following considerations: the nucleophilicity of the amino group of an α,β-dehydro amino acid is weaker than those of a standard amino acid,[41] and a folded conformation of the peptide, required to achieve the cyclization, could be less accessible in the case of a dehydropeptide. For all the cyclic pseudopeptides, the resolution of the L and D stereoisomers was performed after the cyclization by reverse-phase HPLC. The attribution of the L (*R* configuration) or the D (*S* configuration) stereochemistry of the pseudophenylalanine residue was determined by NMR (NOE analysis), according to the procedure of Yamasaki et al.[42]

Cyclo[Gly^1-Pro^2-Phe^{3}Ψ{PO(OH)CH_2}Gly^4-Pro^5-Ahx^6], I. (a) Z-Gly^1-Pro^2-Phe^{3}Ψ{PO(OH)CH_2}Gly^4-Pro^5-Ahx^6-$NHNH_2$: Z-Gly^1-Pro^2-Phe^{3}Ψ{PO(OH)CH_2}Gly^4-Pro^5-Ahx^6-OCH_3 (0.045 g, 0.058 mmol), prepared as described previously,[16] was dissolved in methanol (2.5 mL), and then anhydrous hydrazine (0.6 mL, 18.5 mmol) was added. After 20 h, the reaction mixture was evaporated. The residue was dissolved in methanol and reevaporated. This was repeated twice. This residue was dissolved in a small volume of methanol and precipitated by adding a mixture of diethyl ether/petroleum ether (1:2), filtered, washed, and dried to give the protected hydrazide in 87% yield; R_f (1), 0.34; R_f (2), 0.13.

(b) H-Gly^1-Pro^2-Phe^{3}Ψ{PO(OH)CH_2}Gly^4-Pro^5-Ahx^6-$NHNH_2$: The above compound (0.035 g, 0.0438 mmol) was dissolved in 95% ethanol (8 mL) and was hydrogenated for 3.5 h in the presence of 10% Pd/C catalyst (0.015 g). The catalyst was removed by filtration, and the filtrate was evaporated to

Table 3. Characterization of the Cyclic Peptides **I–VII**

cyclic peptide no.	formula	FAB-MS (M + H) calcd	FAB-MS (M + H) found	HPLC[a] t_R (min)	TLC (R_f)[b] 1	TLC (R_f)[b] 2
Ia	$C_{29}H_{42}N_5O_7P$	604	604	20[1]	0.28	0.67
Ib			604	22[1]	0.27	0.65
IIa	$C_{23}H_{31}N_4O_6P$	491	491	30.6[2]	0.31	0.74
IIb			491	29.2[2]	0.30	0.68
IIIa	$C_{29}H_{41}N_6O_8P$	633	633	20[2]	0.30	0.76
IIIb			633	22.2[2]	0.27	0.74
IVa	$C_{30}H_{42}N_5O_9PS_2$	712	712	16.6[1]	0.37	0.79
IVb			712	15.8[1]	0.31	0.77
Va	$C_{30}H_{44}N_5O_7P$	618	618	16[1]	0.39	0.80
Vb[c]						
VIa	$C_{30}H_{44}N_5O_8P$	634	634	16[1]	0.18	0.80
VIb			634	17.5[1]	0.19	0.76
VIIa	$C_{30}H_{42}N_5O_7P$	617	617	21.6[1]	0.26	0.82
VIIb[c]						

[a] See text the definition of the analytical HPLC gradients 1 and 2. [b] See text for definition of the solvent systems. [c] Uncharacterized fractions.

dryness. The solid product was triturated with diethyl ether, filtered, washed, and dried to afford the title compound in 90% yield; R_f (3), 0.57; R_f (4), 0.52; R_f (5), 0.58.

To a cold solution (−18 °C) containing the above product (0.018 g, 0.028 mmol) in dimethylformamide (0.2 mL) were successively added 0.1 mL of HCl (1 M), 0.1 mL of glacial acetic acid and 0.056 mL of $NaNO_2$ (0.056 mmol). After ca. 20 min at −10 °C, the reaction mixture was diluted by the addition of cold dimethylformamide (35 mL). The pH of the solution was kept slightly alkaline by dropwise addition of triethylamine. After 24 h at 4 °C, the solvents were removed in vacuo and the residue was dissolved in water (1 mL). The pH of the solution was adjusted to 1.5 by adding 1 M HCl, then this solution was saturated with sodium chloride and finally extracted twice with ethyl acetate and once with chloroform. The organic layer was washed with brine, dried over Na_2SO_4, and concentrated to dryness. The residue was dissolved in chloroform (0.5 mL), precipitated by adding diethyl ether, filtered, washed with diethyl ether, and dried to afford 8 mg of the cyclic peptide (45% yield). The two diastereoisomers were separated by preparative HPLC using the gradient system (1), described in the general procedure, t_R = 22 min (fraction a) and 24 min (fraction b). See Table 3 for analytical data of the final cyclic peptides. ^{1}H NMR (H_2O) fraction a: CH_ϵ Ahx δ 0.87 (t, 3); CH_β Pro^2 δ 1.09 (m, 1); CH_γ Ahx δ 1.25 (m, 2); CH_δ Ahx δ 1.31 (m, 2); CH_α (PO_2CH_2) δ 1.58 (m, 1); CH_β Ahx δ 1.62 (m, 1); CH_γ Pro^2 δ 1.75 (m, 1); CH_β Ahx δ 1.85 (m, 1); CH_γ Pro^2 δ 1.88 (m, 1); CH_β Pro^2 δ 1.92 (m, 1); CH_α (PO_2CH_2) δ 1.95 (m, 1); CH_β Pro^5 δ 1.96 (m, 1); CH_γ Pro^5 δ 2.05 (m, 2); CH_β Pro^5 δ 2.37 (m, 1); CH_α Gly^4 δ 2.55 (m, 1); CH_β Phe^3 δ 2.69 (m, 1, $^2J_{H\beta H\beta'}$ 14, $^3J_{H\alpha H\beta}$ 11.6, $^3J_{H\alpha P}$ 6.6); CH_α Gly^4 δ 2.81 (m, 1); $CH_{\beta'}$ Phe^3 δ 3.29 (dd, 1, $^2J_{H\beta H\beta'}$ 14, $^3J_{H\alpha H\beta e}$ 3.0); CH_δ Pro^2 δ 3.43 (m, 1); CH_δ Pro^2-Pro^5 δ 3.67 (m, 2); CH_δ Pro^5 δ 3.90 (m, 1); CH_α Gly^1 δ 3.91 (dd, 1, $^2J_{\alpha\alpha'}$ 17, $^3J_{NH/H\alpha}$ 5.7); CH_α Pro^2 δ 4.12 (m, 1); $CH_{\alpha'}$ Gly^1 δ 4.21 (dd, 1, $^2J_{\alpha\alpha'}$ 17, $^3J_{NH/H\alpha'}$ 4.8); CH_α Phe^3 δ 4.31 (m, 1); CH_α Pro^5 δ 4.37 (m, 1); CH_α Ahx δ 4.53 (m, 1); Ar Phe^3 δ 7.25–7.35 (m, 5); NH Phe^3 δ 7.57 (d, 1, $^3J_{NH/H\alpha}$ 10.3); NH Ahx δ 8.02 (d, 1, $^3J_{NH/H\alpha}$ 9.0); NH Gly^1 δ 8.12 (dd, 1).

Fraction b: CH_ϵ Ahx δ 0.85 (t, 3); CH_β Pro^2 δ 1.12 (m, 1); CH_γ Ahx δ 1.22 (m, 2); CH_δ Ahx δ 1.28 (m, 2); CH_β Ahx δ 1.60 (m, 1); CH_α (PO_2CH_2) δ 1.62 (m, 1); CH_γ Pro^2 δ 1.82 (m, 2); CH_β Ahx δ 1.89 (m, 1); CH_β Pro^5 δ 1.93 (m, 1); CH_β Pro^2 δ 1.99 (m, 1); CH_γ Pro^5 δ 2.04 (m, 2); CH_α (PO_2CH_2) δ 2.24 (m, 1); CH_β Pro^5 δ 2.31 (m, 1); CH_α Gly^4 δ 2.61 (m, 2); CH_β Phe^3 δ 2.74 (m, 1, $^2J_{H\beta H\beta'}$ 14, $^3J_{H\alpha H\beta}$ 13.3, $^3J_{H\alpha P}$ 6.3); $CH_{\beta'}$ Phe^3 δ 3.22 (dd, 1, $^2J_{H\beta H\beta'}$ 14, $^3J_{H\alpha H\beta'}$ 2.0); CH_δ Pro^2 δ 3.35 (m, 1); CH_δ Pro^2 δ 3.52 (m, 1); CH_α Gly^1 δ 3.56 (dd, 1, $^2J_{H\alpha H\alpha'}$ 17, $^3J_{NH/H\alpha}$ 3.0); CH_δ Pro^5 δ 3.60 (m, 1); CH_δ Pro^5 δ 3.76 (m, 1); CH_α Pro^2 δ 4.22 (m, 1); CH_α Phe^3 δ 4.26 (m, 1); CH_α Pro^5 δ 4.32 (m, 1); $CH_{\alpha'}$ Gly^1 δ 4.35 (dd, 1, $^2J_{H\alpha H\alpha'}$ 17, $^3J_{NH/H\alpha'}$ 7.5); CH_α Ahx δ 4.43 (m, 1); Ar Phe^3 δ 7.23–7.38 (m, 5); NH Gly^1 δ 7.75 (dd, 1); NH Ahx δ 8.23 (d, 1, $^3J_{NH/H\alpha}$ 8.7); NH Phe^3 δ 8.33 (d, 1, $^3J_{NH/H\alpha}$ 10.2). ^{31}P NMR (H2O) fraction a, 40.89; fraction b, 43.79. Analysis of the NOE data, according to the procedure of Yamasaki et al.,[42] reveals that the pseudophenylalanine residue in fraction a has a L stereochemistry (*R* configuration), while this residue has a D stereochemistry (*S* configuration) in fraction b.

The following compounds (**II**, **III**, **V**, and **VI**) were prepared and characterized using the procedure described above for the compound **I**.

Cyclo[Gly^1-Pro^2-Phe$^3\Psi${PO(OH)CH$_2$}Gly^4-Pro^5], II. Z-Gly^1-Pro^2-Phe$^3\Psi${PO(OH)CH$_2$}Gly^4-Pro^5-OCH_3 (0.13 g, 0.2 mmol) was converted to the corresponding hydrazide (95% yield); R_f (1), 0.25; R_f (2), 0.31. This protected hydrazide was hydrogenated to give the unprotected hydrazide in 85% yield, R_f (3), 0.32. After the cyclization, the title compound was obtained in 35% yield. The two diastereoisomers were separated by HPLC using the gradient system (2), t_R = 32.6 min (fraction a) and 31.1 min (fraction b). See Table 3 for analytical data of the final cyclic peptides. ^{1}H NMR (H_2O), fraction b: CH_α (PO_2CH_2) δ 1.51 (m, 1); CH_β Pro^2 δ 1.53 (m, 1); CH_β Pro^5-CH_α (PO_2CH_2) δ 1.78 (m, 2); CH_γ Pro^2 δ 1.85 (m, 2); CH_β Pro^5 δ 1.97 (m, 1); CH_β Pro^2 δ 2.04 (m, 1); CH_γ Pro^5 δ 2.18 (m, 1); CH_α Gly^4 δ 2.32 (m, 1); CH_γ Pro^5 δ 2.39 (m, 1); CH_α Gly^4 δ 2.51 (m, 1); CH_β Phe^3 δ 2.72 (m, 1, $^2J_{H\beta H\beta'}$ 14, $^3J_{H\alpha H\beta}$ 13.3, $^3J_{H\alpha P}$ 5.8); $CH_{\beta'}$ Phe^3 δ 3.26 (dd, 1, $^2J_{H\beta H\beta'}$ 14, $^3J_{H\alpha H\beta'}$ 2.0); CH_δ Pro^5 δ 3.50 (m, 2); CH_δ Pro^2 δ 3.52 (m, 1); CH_δ Pro^2 δ 3.65 (m, 1); CH_α Gly^1 δ 3.74 (dd, 1, $^2J_{\alpha\alpha'}$ 17, $^3J_{NH/H\alpha}$ 5.8); CH_α Phe^3 δ 4.10 (m, 1); $CH_{\alpha'}$ Gly^1 δ 4.12 (dd, 1, $^2J_{\alpha\alpha'}$ 17, $^3J_{NH/H\alpha'}$ 5.8); CH_α Pro^2 δ 4.32 (m, 1); CH_α Pro^5 δ 4.82 (m, 1); Ar Phe^3 δ 7.24–7.37(m, 5); NH Phe^3 δ 8.19 (d, 1, $^3J_{NH/H\alpha}$ 10.8); NH Gly^1 δ 8.69 (t, 1). ^{31}P NMR (H_2O) fraction b, 43.79. Analysis of the NOE data reveals that the pseudophenylalanine residue in fraction a has a D stereochemistry.

Cyclo[Gly^1-Pro^2-Phe$^3\Psi${PO(OH)CH$_2$}Gly^4-Ala^5-Pro^6-Ala^7], III. Z-Gly^1-Pro^2-Phe$^3\Psi${PO(OH)CH$_2$}Gly^4-Ala^5-Pro^6-Ala^7-OCH_3 (0.12 g, 0.15 mmol) was treated as described above to give first the corresponding hydrazide (95% yield; R_f (1), 0.25; R_f (2), 0.2). After hydrogenation and cyclization, the title compound was obtained in 45% yield. The two diastereoisomers were separated by HPLC using the gradient system (2), t_R = 23 min (fraction a) and 25 min (fraction b). See Table 3for analytical data of the final cyclic peptides. ^{1}H NMR (H_2O), fraction a: CH_3 Ala^5 δ 1.33 (d, 3, $^3J_{CH_3H\alpha}$ 7.0); CH_β Pro^2 δ 1.37 (m, 1); CH_α (PO_2CH_2) δ 1.49 (m, 1); CH_3 Ala^7 δ 1.50 (d, 3, $^3J_{CH_3H\alpha}$ 7.3); CH_α (PO_2CH_2) δ 1.72 (m, 1); CH_γ Pro^6 δ 1.78 (m, 1); CH_β Pro^2 δ 1.95 (m, 1); CH_γ Pro^6 δ 2.00 (m, 1); CH_γ Pro^2 δ 2.02 (m, 2); CH_α Gly^4 δ 2.20 (m, 1); CH_β Pro^6 δ 2.24 (m, 1); CH_β Pro^6 δ 2.33 (m, 1); CH_α Gly^4 δ 2.41 (m, 1); CH_β Phe^3 δ 2.64 (m, 1, $^2J_{H\beta H\beta'}$ 14, $^3J_{H\alpha H\beta}$ 13.1, $^3J_{H\alpha P}$ 6.2); $CH_{\beta'}$ Phe^3 δ 3.20 (dd, 1, $^2J_{H\beta H\beta'}$ 14, $^3J_{H\alpha H\beta'}$ 2.5); CH_δ Pro^2 δ 3.43 (m, 1); CH_δ Pro^6 δ 3.53 (m, 1); CH_δ Pro^6 δ 3.66 (m, 1); CH_δ Pro^2 δ 3.71 (m, 1); CH_α Pro^2 δ 4.01 (m, 1; $CH_{\alpha\alpha'}$ Gly^1 δ 4.13 (ABX, 2, $^2J_{\alpha\alpha'}$ 17); CH_α Ala^5 δ 4.18 (dd, 1); CH_α Phe^3 δ 4.25 (m, 1); CH_α Ala^7 δ 4.39 (t, 1); CH_α Pro^5 δ 4.68 (m, 1); Ar Phe^3 δ 7.24–7.37 (m, 5); NH Gly^1 δ 7.58 (t, 1, $^3J_{NH/H\alpha}$ 4.4); NH Phe^3 δ 7.83 (dd, 1, $^3J_{NH/H\alpha}$ 10.3, $^3J_{NH/P}$ 2.4); NH Ala^7 δ 8.33 (d, 1, $^3J_{NH/H\alpha}$ 7.0); NH Ala^5 δ 8.54 (d, 1, $^3J_{NH/H\alpha}$ 2.0). ^{31}P NMR (H_2O) fraction a, 40.84. Analysis of the NOE data reveals that the pseudophenylalanine residue in fraction a has an L stereochemistry.

Cyclo[βAla^1-Pro^2-Phe$^3\Psi${PO(OH)CH$_2$}Gly^4-Pro^5-Ahx^6], V. Boc-βAla^1-Pro^2-Phe$^3\Psi${PO(OH)CH$_2$}Gly^4-Pro^5-Ahx^6-OCH_3 (0.013 g, 0.017 mmol) was converted to the corresponding hydrazide (96% yield, R_f (2), 0.5). The Boc group was removed by 4 M HCl/dioxane to afford the hydrochloride salt of H-βAla^1-Pro^2-Phe$^3\Psi${PO(OH)CH$_2$}Gly^4-Pro^5-Ahx^6-$NHNH_2$ (95% yield; R_f (1), 0.18; R_f (2), 0.12). After the cyclization the title compound was obtained in 35% yield. Only one of the two diastereoisomers was recovered by HPLC under pure state using the gradient system (1), t_R = 18 min (fraction a). See Table 3 for analytical data of the final cyclic peptide. ^{1}H NMR (H_2O), fraction a: CH_ϵ Ahx δ 0.88 (t, 3); CH_α (PO_2CH_2) δ 1.23 (m, 1); CH_γ Ahx-CH_δ Ahx δ 1.34 (m, 4); CH_γ Pro^2 δ 1.38 (m, 1); CH_β Pro^2 δ 1.55 (m, 1); CH_β Pro^5 δ 1.63 (m, 1); CH_β Ahx δ 1.74 (m, 1); CH_α (PO_2CH_2)-CH_α Gly^4 δ 1.76 (m, 2); CH_γ Pro^2 δ 1.78 (m, 1); CH_β Ahx δ 1.83 (m, 1); CH_γ Pro^5 δ 1.85 (m, 1); CH_γ Pro^5 δ 1.92 (m, 1); CH_β Pro^2 δ 1.98 (m, 1); CH_β Pro^5 δ 2.26 (m, 1); CH_α Gly^4 δ 2.52 (m, 1); CH_β β-Ala1 δ 2.57 (m, 2); CH_β

Phe^3 δ 2.71 (m, 1, $^2J_{H\beta H\beta'}$ 14, $^3J_{H\alpha H\beta}$ 13.3, $^3J_{H\alpha P}$ 6.5); $CH_{\beta'}$ Phe^3 δ 3.28 (dd, 1, $^2J_{H\beta H\beta'}$ 14, $^3J_{H\alpha H\beta'}$ 2.8); CH_α β-Ala1 δ 3.31 (m, 2); CH_δ Pro^5 δ 3.35 (m, 2); CH_δ Pro^2 δ 3.48 (m, 1); CH_δ Pro^2 δ 3.56 (m, 1); CH_α Pro^2-CH_α Ahx δ 4.22 (m, 2); CH_α Phe^3 δ 4.30 (m, 1); CH_α Pro^5 δ 4.33 (m, 1); NH β-Ala^1 δ 6.86 (t, 1); Ar Phe^3 δ 7.24–7.37 (m, 5); NH Phe^3 δ 7.81 (d, 1, $^3J_{NH/H\alpha}$ 10.3); NH Ahx δ 8.37 (d, 1). ^{31}P NMR (H_2O) fraction a, 40.63. Analysis of the NOE data reveals that the pseudophenylalanine residue in fraction a has an L stereochemistry.

Cyclo[Ser^1-Pro^2-Phe$^3\Psi${PO(OH)CH$_2$}Gly^4-Pro^5-Ahx^6], VI. Boc(OBzl)-Ser^1-Pro^2-Phe$^3\Psi${PO(OH)CH$_2$}Gly^4-Pro^5-Ahx^6-OCH_3 (0.116 g, 0.184 mmol) was converted to the corresponding hydrazide (95% yield, R_f (1), 0.51 and 0.61; R_f (2), 0.7 and 0.79, respectively, for the two diastereoisomers). The Boc group was removed as usual to give the hydrochloride salt of hydrazide in 90% yield; R_f (2), 0.45 and 0.45; R_f (3), 0.71 and 0.74, respectively, for the two diastereoisomers. Cyclization of the partial protected hydrazide gave the compound cyclo-[Ser^1(OBzl)-Pro^2-Phe$^3\Psi${PO(OH)CH$_2$}Gly^4-Pro^5-Ahx^6] in 55% yield. R_f (1), 0.43; R_f (2), 0.66 and 0.73 for the two diastereoisomers. This compound was hydrogenated to afford the title compound in 83% yield. The two diastereoisomers were separated by HPLC using the gradient system (1), t_R = 8 min (fraction a) and 10.2 min (fraction b). See Table 3for analytical data of the final cyclic peptides.

Cyclo[ΔAla^1-Pro^2-Phe$^3\Psi${PO(OH)CH$_2$}Gly^4-Pro^5-Ahx^6], VII. To a cold solution containing compound **VI** (0.04 g, 0.06 mmol) in pyridine (0.8 mL) was added methanesulfonyl chloride (0.02 g, 0.1 mmol). After 24 h at room temperature the solvents were removed in vacuo, the residue was dissolved in water (1 mL) and acidified to pH 2 with 1 M $NaHSO_4$ and the aqueous phase was extracted twice with ethyl acetate. The organic layer was dried with Na_2SO_4 and concentrated to dryness. The residue was dissolved in small volume of chloroform, precipitated by adding petroleum ether, filtered, washed, and dried to give the compound cyclo[Ser^1(OMS)-Pro^2-Phe$^3\Psi${PO(OH)CH$_2$}Gly^4-Pro^5-Ahx^6 in 68% yield.

To a cold solution of the above cyclic peptide (0.030 g, 0.04 mmol) in dichloromethane (0.2 mL) was added triethylamine (0.2 mL, 0.16 mmol) dropwise. After 1 h at 0 °C and 1 h at room temperature, the reaction mixture was concentrated to dryness. The residue was dissolved in water (1 mL), acidified to pH 2 with 1 M $NaHSO_4$ and the product which precipitated out was extracted with chloroform. The chloroformic solution was dried with Na_2SO_4 and evaporated to dryness. The residue was dissolved in small volume of chloroform, and the product was precipitated by adding petroleum ether, filtered, washed, and dried to give the title compound in 25% yield. Only one of the two diastereoisomers was recovered by HPLC under pure state using the gradient system (1), t_R = 23.6 min (fraction a). See Table 3 for analytical data of the final cyclic peptide.

Ac-cyclo[Cys^1-Pro^2-Phe$^3\Psi${PO(OH)CH$_2$}Gly^4-Pro^5-Cys^6]-OCH3, IV. (a) Boc-Cys^1-Pro^2-Phe$^3\Psi${PO(OH)CH$_2$}Gly^4-OCH_2-CH_3: Boc-Pro-PheΨ{PO(OH)CH$_2$}Gly-OCH_2CH_3 (0.25 g, 0.52 mmol) was treated with 4 M HCl/dioxane (2 mL) to remove the Boc group. Then this hydrochloride salt was triturated with diethyl ether, filtered, and dried. To a solution of this hydrochloride salt (0.1 g, 0.239 mmol) in dimethylformamide (2 mL) was added triethylamine (0.067 mL, 0.48 mmol), followed by the addition of Boc-Cys(Acm)-ONph (0.107 g, 0.26 mmol). The reaction mixture was kept slightly alkaline (pH 8) by addition of triethylamine. After 24 h, the solvents were removed in vacuo, and the residue was dissolved in saturated solution of $NaHCO_3$ (5 mL). The aqueous solution was extracted with ethyl acetate and then acidified with 2 M HCl, and the precipitate was immediately extracted with ethyl acetate, washed with brine, dried over Na_2SO_4, and concentrated to dryness to give the title compound as an oil in 95% yield: R_f (1), 0.58; R_f (2), 0.73

(b) Boc-Cys^1(Acm)-Pro^2-Phe$^3\Psi${PO(OH)CH$_2$}Gly^4-OH: The above compound (0.15 g, 0.228 mmol) was dissolved in a mixture containing water (2 mL) and 4 M NaOH (0.2 mL). After 1.5 h at room temperature, the reaction mixture was acidified with 1 M $NaHSO_4$. The precipitated product was taken up in ethyl acetate, and the organic phase was washed with water, dried over Na_2SO_4, and concentrated to dryness. The residue was dissolved in small volume of chloroform (2 mL), precipitated by adding petroleum ether, filtered and dried to afford the title compound in 80% yield: R_f (1), 0.43; R_f (2), 0.46.

(c) Boc-Cys^1(Acm)-Pro^2-Phe$^3\Psi${PO(OH)CH$_2$}Gly^4-Pro^5-Cys^6 (Acm)-OCH_3: To a chilled solution of the above compound (0.11 g, 0.175 mmol) in anhydrous tetrahydrofuran (1 mL) was added 1,1-carbonyldiimidazole (0.068 g, 0.42 mmol). After 40 min at room temperature, H-Pro-Cys(Acm)-OCH_3, dissolved in a mixture of tetrahydrofuran/dimethylformamide, 1:1 (0.6 mL), was added to the reaction mixture. After 5 h at room temperature, the solvents were removed in vacuo. The residue was dissolved in water (5 mL) and acidified to pH 2 with 1 M HCl. The precipitated product was immediately taken up in ethyl acetate. The organic layer was washed with brine, dried over Na_2SO_4, and evaporated to dryness. The residue was dissolved in chloroform (2 mL) and precipitated by adding petroleum ether, filtered, washed, and dried to afford the title compound in 80% yield: R_f (2), 0.47.

(d) Boc-cyclo[Cys^1-Pro^2-Phe$^3\Psi${PO(OH)CH$_2$}Gly^4-Pro^5-Cys^6]-OMe: To a solution of iodine (0.35 g, 1.35 mmol) in methanol (70 mL),[43] was added dropwise the above compound (0.11 g, 0.135 mmol) in methanol (80 mL) (1 h duration). After 45 min at room temperature, a saturated solution of $NaHCO_3$ (1 mL) was added to the reaction mixture, and the excess of iodine was destroyed with 1 M solution of sodium thiosulfate. The solvents were removed in vacuo, and the residue was treated with water (3 mL), acidified to pH 2 with 1 M $NaHSO_4$ and immediately taken up in ethyl acetate. The organic phase was washed with brine, dried over Na_2SO_4, and evaporated to dryness. The new residue was treated with diethyl ether, filtered, washed, and dried to give the title compound **IV** in 50% yield.

(e) Ac-cyclo[Cys^1-Pro^2-Phe$^3\Psi${PO(OH)CH$_2$}Gly^4-Pro^5-Cys^6]-OMe: The Boc group was removed with 4 N HCl/dioxane as described above. Then, the N-terminal amino group was acetylated with acetic anhydride (9.5 μL, 0.1 mM) in pyridine (1 mL). After the solvents were removed under vacuum, the residue was dissolved in ethyl acetate (5 mL), the organic layer was washed two times with 1 M $NaHSO_4$, dried over Na_2SO_4, and concentrated to dryness. The residue was then triturated in diethyl ether and filtered to give the title compound **V** in pure form (85% yield). The two diastereoisomers were separated by HPLC using the gradient system (1), t_R = 18.6 min (fraction a) and 17.8 min (fraction b). See Table 3 for analytical data of the final cyclic peptides. ^{1}H NMR (H_2O), fraction b: CH_β Pro^2 δ 1.12 (m, 1) ; CH_α (PO_2CH_2) δ 1.60 (m, 1) ; CH_γ Pro^2 δ 1.85 (m, 2) ; CH_β Pro^2 δ 1.92 (m, 1) ;CH_β Pro^5 δ 2.00 (m, 1); CH_α (PO_2CH_2) δ 2.03 (m, 1); CH_γ Pro^5 δ 2.04 (m, 2) ; CH_3(CO) δ 2.06 (s, 3) ; CH_β Pro^5 δ 2.28 (m, 1); CH_α Gly^4 δ 2.58 (m, 1); CH_β Cys^1 δ 2.65 (m, 1, $^2J_{H\beta H\beta'}$ 14, $^3J_{H\alpha H\beta}$ 11.7); CH_α Gly^4 δ 2.68 (m, 1); CH_β Phe^3 δ 2.76 (m, 1, $^2J_{H\beta H\beta'}$ 14, $^3J_{H\alpha H\beta}$ 13.3, $^3J_{H\alpha P}$ 6.1); CH_β Cys^6 δ 2.92 (dd, 1, $^2J_{H\beta H\beta'}$ 14, $^3J_{H\alpha H\beta}$ 11.5); $CH_{\beta'}$ Phe^3 δ 3.23 (dd, 1, $^2J_{H\beta H\beta'}$ 14, $^3J_{H\alpha H\beta'}$ 2.1); $CH_{\beta'}$ Cys^6 δ 3.29 (dd, 1, $^2J_{H\beta H\beta'}$ 14, $^3J_{H\alpha H\beta'}$ 2.9); $CH_{\beta'}$ Cys^1 δ 3.38 (dd, 1, $^2J_{H\beta H\beta'}$ 14, $^3J_{H\alpha H\beta'}$ 2.3); CH_δ Pro^2 δ 3.45 (m, 1); CH_δ Pro^2 δ 3.52 (m, 1); CH_δ Pro^5 δ 3.64 (m, 2); CH_3(O-CO) δ 3.76 (s, 3); CHα Pro^2 δ 4.15 (m, 1); CH_α Phe^3 δ 4.19 (m, 1); CH_α Pro^5 δ 4.45 (m, 1); CH_α Cys^1 δ 4.75 (m, 1); CH_α Cys^6 δ 4.80 (m, 1); Ar Phe^3 δ 7.25–7.37(m, 5); NH Phe^3 δ 8.28 (d, 1, $^3J_{NH/H\alpha}$ 10.0); NH Cys^1 δ 8.63 (d, 1, $^3J_{NH/H\alpha}$ 8.4); NH Cys^6 δ 8.94 (d, 1, $^3J_{NH/H\alpha}$ 8.0). ^{31}P NMR (H_2O) fraction b, 42.98. Analysis of the NOE data reveals that the pseudophenylalanine residue in fraction a has a D stereochemistry.

Inhibition Constants. Collagenase from *C. rathayii*[7] was purified from a crude extract in a two steps procedure. This material (1 mg/mL) was applied on a gel filtration column (TSK SW3000, Toyosoda) equilibrated with Tricine–NaOH buffer 50 mM pH 7, CaCl2 10 mM, NaCl 50 mM. Further purification was achieved by FPLC on anion-exchange column (MonoQ, Pharmacia). The enzyme was eluted by applying a NaCl gradient (0.05–1 M). The major peak, containing the activity against the furanacryloyl-Leu-Gly-Pro-Ala substrate, when analyzed by SDS/PAGE, shows a single band at the molecular range of 81 kDa.

All assays were performed under the following buffer conditions: 25 °C, pH 7.5, 50 mM Tricine–NaOH, 10 mM CaCl2, 1 M NaCl, and 1.25 mg/mL of 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 a substantial stabilization of the collagenase activity, without affecting the kinetic properties of the enzyme. Under this condition, 0.1–1 nM samples of collagenase retain almost their original activity at 25 °C during 4 days. Collagenase activity was essayed using the synthetic substrate furanacryloyl-Leu-Gly-Pro-Ala, as described by Van Wart and Steinbrink.[44] The concentration of the enzyme was determined spectrophotometrically using $\epsilon_{280} = 1.5 \times 10^5\ M^{-1}\ cm^{-1}$.

Slow binding inhibitors impose sometime long equilibrium times such that the equilibrium cannot be observed before a complete depletion of the substrate. In this case, the measurements were conducted by equilibrating increasing concentrations of inhibitor in a 2-mL solution containing 0.1–1 nM of enzyme for one night, and then initiating the reaction by adding 10 μL of substrate to determine the residual free enzyme concentration. In the view of the slow dissociation rates for most of the inhibitors, the influence of the addition of a competitive substrate on the position of the equilibrium could be neglected. The determination of the position of the equilibrium allows the calculation of the K_i value. The determination of the k_{on} values was performed as previously described.[16] All the values reported for K_i and k_{on} were reproducible within ±10% and ±15%, respectively.

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