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Cyclopeptidic inactivators for chymotrypsin-like proteinases

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Summary — Cyclopeptides containing a functionalized *meta*-aminobenzoic acid residue (*m*-aB[CH_2X] with X = OC_6H_5, OAc, Br, Cl) linked to a tetraglycyl–phenylalanyl sequence, have been synthesized in solution. A phenyl ether group has been used during chain elongation and cyclisation, and then cleaved by treatment with HBr/HOAc to give the corresponding bromide, from which the acetate and the chloride have been obtained. The functionalized aminobenzoic acid residues possess a latent quinonimmonium methide electrophilic function, and these cyclopeptides are potential "suicide" substrates of chymotrypsin-like proteases. The cyclopeptides with X = Br or Cl irreversibly inhibit α-chymotrypsin (k_{inact}/K_I = 180 $M^{-1}min^{-1}$ for X = Cl). The compounds with poorer leaving groups X = OAc or OC_6H_5 are devoid of inactivating effect and only behave as substrates of this enzyme (k_{cat}/K_M = 31 800 $M^{-1}min^{-1}$ and 120 000 $M^{-1}min^{-1}$, respectively).

Résumé — Inactivateurs cyclopeptidiques de protéinases de spécificité chymotrypsique. *Des cyclopeptides contenant un reste d'acide* meta-*aminobenzoïque fonctionnalisé* (m-*aB[CH_2X] avec X = OC_6H_5, OAc, Br, Cl) lié à une séquence tétraglycyl-phénylalanine ont été préparés par synthèse peptidique en solution. Un groupement éther de phényle a été employé durant l'élongation de la chaîne et la cyclisation. En fin de synthèse, ce groupement éther a été coupé par acidolyse (HBr/HOAc) pour donner un bromure à partir duquel les acétate et chlorure correspondants ont été obtenus. Ces cyclopeptides sont des substrats « suicide » potentiels des protéases de spécificité chymotrypsique car le reste acide* méta-*aminobenzoïque fonctionnalisé possède une fonction électrophile latente méthylène quinonimmonium. Les cyclopeptides dans lesquels X = Br ou Cl sont des inactivateurs de l'α-chymotrypsine (k_{inact}/K_I = 180 $M^{-1}min^{-1}$ pour X = Cl). Lorsque X est un moins bon groupe partant (X = OAC ou OC_6H_5), ces composés n'ont pas d'effet inactivateur et se comportent comme des substrats de l'enzyme (k_{cat}/K_M = 31 800 $M^{-1}min^{-1}$ et 120 000 $M^{-1}min^{-1}$ et respectivement).

α-chymotrypsin / cyclopeptides / *meta*-aminobenzoic acid / quinonimmonium methide / serine proteases / "suicide" substrates

Introduction

Chymotrypsin-like proteinases play important roles in normal and pathological processes. Cathepsin G can assist neutrophil elastase in the uncontrolled proteolysis of components of connective tissue implicated in the etiology of inflammatory diseases such as pulmonary emphysema [1] and can produce angiotensin I from kininogen [2]. Mast cell chymase may participate in the lesions that characterize psoriasis and may enhance allergic responses [3]. Therefore, the selective inhibition of chymotrypsin-like proteinases is of therapeutic interest.

"Suicide" substrates are expected to display a maximum selectivity towards their target enzyme since their inhibitory activities require discrimination in binding steps, catalytic activation by the enzyme and irreversible modification of the active center [4]. Previously, we synthesized 6-halomethyl-3,4-dihydrocoumarins [5–7], which proved to be the first efficient general suicide substrates for proteases [5, 8–11]. However, the selectivity of the inhibition was poor. 6-Chloromethyl-3-benzyl-3,4-dihydro-quinolinone, an aza analogue of these dihydrocoumarins which possess a latent electrophilic quinonimmonium methide function, failed to inactivate α-chymotrypsin [12]. This result presumably indicates that the molecule, in

Abbreviations: PNA: *p*-nitrophenyl acetate; NPGB: *p*-nitrophenyl *p*-guanidinobenzoate; Z-Ala-Ala-Pro-aza-Ala-Ala-ONp: *N*-benzyloxycarbonyl alanylalanylpropylazaalanylalanine *p*-nitrophenyl ester; Ac-Tyr-pNA: *N*-acetyl-L-tyrosine *p*-nitrophenyl ester; Suc-Ala_3-pNA: succinyl-alanyl-alanyl-alanine *p*-nitroanilide; Bz-Arg-pNA: benzoyl-arginine *p*-nitroanilide; *m*-aB[CH_2X]: 2-CH_2X-5-amino-benzoic acid residue (X = OC_6H_5, Br, Cl or OAc; O_2-*m*-aB[$CH_2OC_6H_5$]: 2-phenoxymethyl-5-nitro-benzoic acid residue

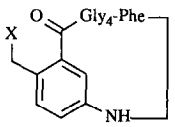
which an *s-cis* amide bond configuration is imposed by the 6-membered lactam ring, does not fulfill the enzyme stereoelectronic requirements to be an effective substrate [12, 13]. In macrocyclic lactams, an *s-trans* configuration of the scissile amide bond may be achieved [11]. Moreover, in cyclic peptides a covalent peptidic sequence may be chosen so as to induce a discrimination among proteases by interacting with the primary binding site S_1 and the affinity subsites (S_2, S_3...) of the target enzyme (Schechter and Berger notation [14]). Thus, as a potential new class of mechanism-based serine proteases inhibitors, the synthesis and the study of functionalized aminobenzoic acid containing cyclopeptides was undertaken. A preliminary work on model cyclopeptides having a tetraglycylphenylalanine sequence, but lacking the latent electrophilic function, showed that the cyclopeptide **1** (fig 1) containing a *meta*-aminobenzoic acid residue was a better substrate of α-chymotrypsin than its *ortho*-analog [15–17]. Therefore, the synthesis of the functionalized cyclopeptides **2–5** was realized starting from a *meta*-aminobenzoic acid derivative, and their potential inhibitory properties towards the model proteinase α-chymotrypsin and other serine proteinases were analyzed.

Results

Chemistry

Because of the insolubility of the *meta* cyclopeptide **1** in the usual organic solvents, functionalization of its methyl group was not attempted. It was preferred to functionalize the methyl group of the starting 2-methyl-5-nitro-benzoic acid, and then to incorporate this functionalized residue in a peptidic fragment by peptide synthesis in solution.

First, 2-methyl-5-nitro-benzoic acid **6** was converted to the corresponding methyl ester **7** (fig 2).



1 : X = H
2 : X = OC_6H_5
3 : X = Br
4 : X = OAc
5 : X = Cl

Fig 1. Structure of the c[Phe-*m*-aB(CH_2X)-Gly_4] cyclopeptides: **1–5**.

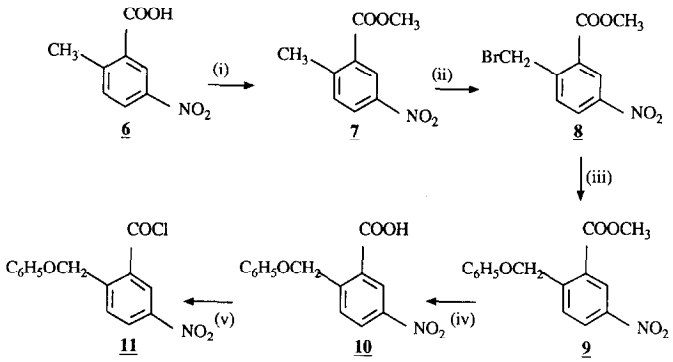


Fig 2. Synthetic scheme for the preparation of the phenoxymethyl substituted *meta*-nitro-benzoylchloride **11** (i) MeOH/CH_3COCl; (ii) NBS/CCl_4; (iii) C_6H_5OH/NaOH/H_2O/CH_2Cl_2/aliquat; (iv) 1 • aq KOH/MeOH $2{\cdot}H_3O^+$; (v) $SOCl_2$/DMF cat.

Bromation by *N*-bromosuccinimide led to the benzylic bromide **8** which was treated with sodium phenoxide in phase transfer conditions [18]. The resulting phenyl ether **9** was easily purified by a single crystallization from the crude product mixture. The ester function was saponified and the acid **10** converted to the acid chloride **11** which was coupled to a tetraglycine ethyl ester fragment to give the peptide **12** (fig 3). Catalytic hydrogenation over platinum oxide allowed the selective reduction of the nitro group to the corresponding amino group, without hydrogenolysis of the benzylic ether. The resulting aromatic amine **13** was unstable, because of the donor effect of the amino group which can lead to elimination of phenol [11]. Thus, this compound was not purified and was directly coupled

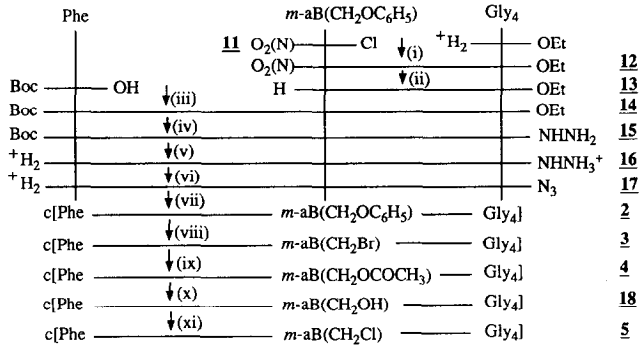


Fig 3. Synthetic scheme for the preparation of the functionalized cyclopeptides **2–5**. (i) Et_3N/DMF; (ii) H_2/PtO_2, DMF-MeOH; (iii) DCC/CH_2Cl_2-DMF; (iv) H_2NNH_2/MeOH; (v) HCl/AcOH; (vi) HCl/THF, RONO/DMF; (vii) iPr_2NEt; (viii) HBr/AcOH; (ix) CH_3COOK/DMF; (x) Et_3N/MeOH; (xi) $SOCl_2$/DMF.

with Boc-L-phenylalanine, activated by dicyclohexylcarbodiimide (DCC). This coupling, realized in dichloromethane with only the minimum quantity of dimethylformamide necessary for solubilization of the amine **13**, was fast enough to occur before side-reactions of decomposition. The obtained peptide **14** was stable and could be purified by chromatographic methods. In view of its cyclization by the azide method, the ester function of **14** was first converted to the corresponding hydrazide function. The terminal amino function of the resulting peptide **15** was then deprotected, leading to the compound **16**. Treatment of **16** by isoamylnitrite in acidic conditions led to the intermediate azide **17** which cyclized *in situ* after dilution and neutralization, to give the cyclopeptide **2**.

Substitution of the phenoxy group of **2** by a bromine atom was realized by means of hydrobromic acid in acetic acid, which are reported conditions for deprotection of *O*-benzyl-tyrosine [19]. The bromide **3** thus obtained could be converted to the corresponding chloride **5** in 3 steps: substitution of the bromine atom by an acetate group on treatment of **3** by potassium acetate in DMF, transesterification of the resulting acetate **4** in methanol/triethylamine, leading to the alcohol **18**, and conversion of this alcohol to the chloride **5** with thionyl chloride in DMF. The chloride **5** was stable in both MeOH or DMF solutions, whereas the corresponding bromide **3** decomposed within a few hours in these solvents.

Enzymology

*Effect of cyclopeptides **1–5** on the activity of serine proteases*

Exposure of α-chymotrypsin (10 μM) to molar excess of **5** ranging from 20–57 at pH 7.5 and 25°C, resulted in a time-dependent loss of hydrolytic activity towards Ac-Tyr-pNA. The inactivation process followed first-order kinetics characterized by the rate constants k_{obs} evaluated by least-squares regression analysis. The reversible or irreversible character of the inhibition was explored at various reaction times after removing the excess inhibitor by filtration–centrifugation at 4°C on Centricon 10 microconcentrator. The inhibition process was found to be irreversible. Upon standing at 4°C for 24 h, the inhibited enzyme regained less than 0.1% enzyme activity. Moreover, addition of buffered hydroxylamine (0.75 M, pH 8.5) to the inhibited enzyme resulted in less than 0.1% reactivation of the filtered enzyme at 4°C. In the presence of **3** (35 μM), chymotrypsin was also irreversibly inhibited. However, only 25% enzyme inhibition was observed in 20 min for a molar excess of $[I]_0/[E]_0$ of 63. This low reactivity prevented an accurate determination of the kinetic parameters. No inactivation of porcine pancreatic elastase, bovine trypsin and human urokinase (a trypsin-like serine protease) was observed after treatment of these enzymes by large molar excesses (> 100) of cyclopeptide **3** or **5** over enzyme.

Cyclopeptides **1**, **2** and **4** when assayed as potential inactivators of α-chymotrypsin by following an analogous procedure, were found to be inefficient. For example, the percentage of activity of chymotrypsin before and after treatment by a 100-fold excess of reagent **1**, **2** or **4** during 45 min, were respectively 100, 99% for **2** and 100, 100% for **1** and **4**. As expected, these cyclopeptides failed to inactivate trypsin, urokinase and elastase.

*Kinetic of inactivation of α-chymotrypsin by **5***

A plot of the inverse of the pseudo-first order rate constant (k_{obs}) *versus* the inverse of the inhibitor concentration yielded a straight line (fig 4). This is consistent with the formation of a reversible complex (E·I) prior to inactivation according to the minimal kinetic scheme (eqn 1) where E is the enzyme, I the inactivator and E–I the inactivated enzyme with the inhibitor covalently attached, and suggests that the equation of Kitz and Wilson [20] is valid (eqn 2).

$$E + I \underset{}{\overset{K_I}{\rightleftharpoons}} E{\cdot}I \xrightarrow{k_{inact}} E\text{–}I \qquad (1)$$

$$1/k_{obs} = K_I/k_{inact} \cdot 1/[I] + 1/k_{inact} \qquad (2)$$

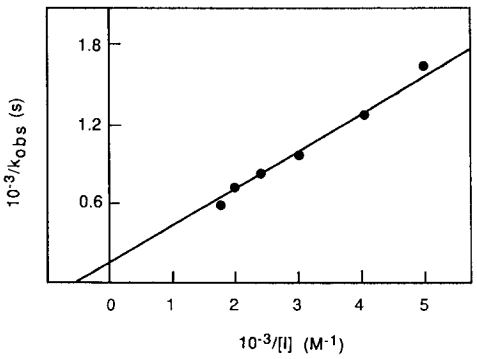


Fig 4. Inactivation of α-chymotrypsin by cyclopeptide **5**. Chymotrypsin (10 μM) was incubated with a 20–57-fold molar excess (over enzyme) of compound **5** at pH 7.5, 25°C, and activity was assayed periodically with Ac-Tyr-pNA. Details are given in *Experimental protocols*. A semi-logarithmic plot of the percentage of remaining enzyme activity *versus* time (not shown) gives a straight line with a slope of $-k_{obs}$. A plot of $1/k_{obs}$ *versus* the inverse of the initial inhibitor concentrations is linear and characterized by a y intercept of $1/k_i$ and an x intercept of $-K_i$.

The apparent dissociation constant K_I and the alkylation rate constant k_{inact} were 2.2 x 10^{-3} M and 0.4 min^{-1}, respectively. The ratio k_{inact}/K_I (180 $M^{-1}min^{-1}$) is the bimolecular constant for the inactivation, and represents the inactivation potency.

Chymotrypsin-catalyzed hydrolysis of cyclopeptides **2** and **4**

When followed spectrophotometrically, evolution with time of the hydrolysis of peptides **2** and **4** (200 µM) catalyzed by α-chymotrypsin (16 µM) at pH 7.5, resulted in a diagram showing the appearance of a new characteristic peak at 292 nm for **2** and 300 nm for **4**. The kinetic parameters were determined at pH 7.5 and 37°C from the intial rates of hydrolysis (fig 5) and were found equal to: k_{cat} = 144 min^{-1}, K_M = 1.2 x 10^{-3} M, k_{cat}/K_M = 120 000 $M^{-1}min^{-1}$ for **2** and k_{cat}/K_M = 31 800 $M^{-1}min^{-1}$ for **4**. In the absence of enzyme, the spontaneous hydrolysis of **2** and **4** was undetectable.

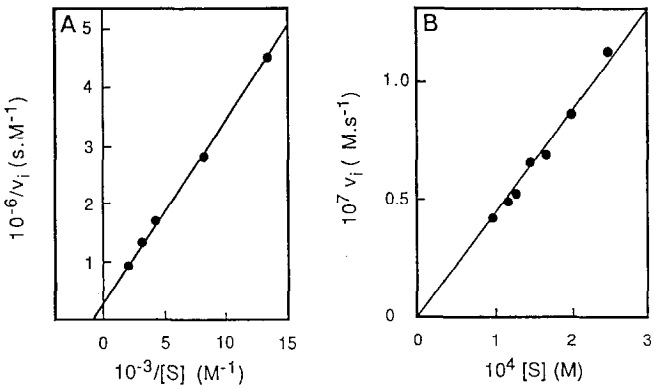


Fig 5. Hydrolysis of cyclopeptides **2** (A) and **4** (B) catalyzed by α-chymotrypsin at pH 7.5 and 37°C. $[S]_0$: cyclopeptide initial concentration; v_i: initial velocity. Enzyme concentration: 1.46 (**2**) and 0.83 µM (**4**).

Discussion

The design of the present inhibitors of α-chymotrypsin is based on the following grounds (fig 6): i), the selective cleavage of the Phe-*m*-aB amide bond induced by the affinity of the phenylalanine residue for the S_1 primary binding site of this enzyme; ii), the simultaneous demasking of a *para*-aminobenzylic derivative possessing a good leaving group X. Activation of such a benzylic function results from the increase in the electron releasing potency of the demasked *p*-amino group (σ+*pNH*$_2$ = –1.47) compared to the starting *p*-amido substituent (σ+*pNHAc* = –0.58) [11]. It is well established that *ortho*- and *para*-aminobenzyl derivatives having a good leaving group X are very reactive towards nucleophilic substitution, which occurs by a dissociative mechanism involving a highly electrophilic quinonimmonium methide intermediate [11]. In the present case, alkylation of an active-site nucleophile by this demasked reactive species is expected to lead to an irreversible inhibition of α-chymotrypsin. As a consequence of the cyclic nature of the inhibitor, the alkylating function is tethered in the active site and its diffusion is prevented during the life-time of the acyl-enzyme.

Four functionalized cyclopeptides **2–5**, possessing different potential leaving groups X, have been synthesized (fig 1). Two of them (**3** and **5**) irreversibly inhibit α-chymotrypsin. The 2 others (**2** and **4**) are devoid of inactivating effect.

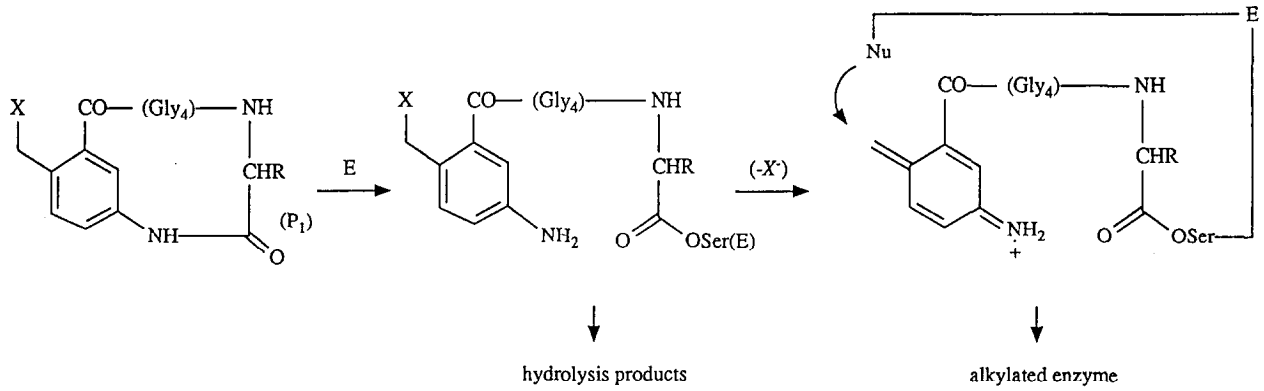


Fig 6. Postulated mechanism for the inactivation of α-chymotrypsin by functionalized cyclopeptides containing a phenylalanine residue (R = $CH_2C_6H_5$).

Inactivation of α-chymotrypsin by the cyclopeptide **5** follows saturation kinetics. When inactivated, the enzyme does not regain activity either after filtration of the treated enzymes on microconcentrator or after treatment with buffered hydroxylamine. This indicates that inactivation is not due to the formation of a stable acyl-enzyme. We previously demonstrated that the cyclopeptide **1** (X = H) (fig 1) is a substrate of α-chymotrypsin, and that the Phe-aB bond is specifically split, in agreement with an attack of the phenylalanine carbonyl group by the active serine [16]. Consequently, a similar selective cleavage may be assumed for the parent functionalized compounds **3** and **5** in agreement with the postulated mechanism (fig 6).

The cyclopeptides **2** and **4** fail to inactivate α-chymotrypsin, but behave as substrates. The cyclopeptide **2** (X = OC_6H_5) is rapidly hydrolyzed by α-chymotrypsin. Compared to **1** (X = H), its k_{cat}/K_M ratio is increased by a factor of *ca* 50. For **4** (X = OAc), this factor is *ca* 10. The only difference between the compounds **2** and **4** and the preceding inhibitors is the replacement of the halide potential leaving group present in **3** and **5** by a phenolate (pK_a = 10.00 at 25°C) [21] in **2** and by an acetate (pK_a = 4.76 at 25°C) [21] in **4**. Thus, a reasonable hypothesis to explain the lack of inactivation is that the rate of elimination of X^- is probably too slow compared to the rate of hydrolysis of the acyl-enzyme, acetate and phenolate being poorer leaving groups than halides.

If compared to halomethylated dihydrocoumarins and other mechanism-based inactivators [4], the functionalized cyclopeptides **3** and **5** are not very potent inhibitors of α-chymotrypsin. However, in contrast with dihydrocoumarins, they already show the expected specificity for α-chymotrypsin *versus* proteinases of elastolytic or tryptic specificity. Since cyclopeptides are subject to easy structural modifications such as change of the leaving group, increase of the ring size and variation of the peptidic sequence, more potent and more selective inactivators may be designed and synthesized, on the basis of the present preliminary study. Concerning the role of the ring size, we have previously observed that c[Phe-*o*-aB-Gly_6] is a better substrate of α-chymotrypsin than its homologous c[Phe-*o*-aB-Gly_4] [5]. Modulation of the peptidic sequence is also of great interest since it is known that interactions with the secondary subsites are of crucial importance for the inhibitory power displayed by peptide inhibitors [3–4]. Therefore, we plan to introduce, besides a P_1 aromatic residue, a $P_2...P_n$ sequence having a good affinity for the $S_2...S_n$ subsites of cathepsin G. Furthermore, by changing the nature of the P_1 residue, these functionalized cyclopeptides are susceptible of being tailored in order to inactivate other classes of serine proteases. The influence of all these structural parameters is now systematically explored in search of a better potency.

Materials and methods

Synthetic procedures

Melting points were recorded on a Tottoli (Büchi) or a Mettler FP61, and are uncorrected. The ^1H NMR measurements were performed on Brücker WH 90 DS, WM 300 or WM 500 instruments. The chemical shifts are reported in ppm, either downfield from tetramethylsilane (TMS) internal standard, or from the attributed chemical shifts of the deuterated solvents: CD_3OD (3.50 ppm), D_2O (4.80 ppm), DMSO–d_6 (2.50 ppm), CD_3COOD (2.10 ppm), as indicated. The coupling constants are given in Hertz. The optical rotations were obtained on a Perkin–Elmer 241 polarimeter. Analyses were performed by the central services of the CNRS. The mass spectrometry measurements were performed by the Departments of JC Tabet (univ Paris VI) and A Gouyette (inst G Roussy). Analytical thin layer chromatography (tlc) and preparative column chromatography were performed on Kieselgel F 254 and on Kieselgel 60, 0.063–0.5 mm (Merck), respectively, with the following solvent systems (by vol): I(x/y): MeOH x%–CH_2Cl_2 y%; II: EtOAc-nBuOH-AcOH-H_2O (1/1/1/1). UV light (254 nm) allowed visualization of the spots after tlc runs for all the compounds, even at low concentration. 2-Methyl-5-nitro-benzoic acid was purchased from Aldrich. The preparation of tetraglycine ethyl ester hydrobromide has been described previously [15].

*O_2-m-aB[$CH_2OC_6H_5$]-OMe (**9**)*

2-Methyl-5-nitro-benzoic acid (**6**) (11.7 g; 63.6 mmol) was esterified to the corresponding methyl ester (**7**) with acetyl chloride in refluxing methanol. The crude methyl ester (**7**) was dissolved in carbon tetrachloride (150 cm^3), *N*-bromosuccinimide (12.5 g; 70 mmol) and benzoyl peroxide (0.15 g) were added, and the mixture was refluxed for 20 h. After cooling and filtration of succinimide over fritted glass, the solution was evaporated *in vacuo*. ^1H NMR (CCl_4; TMS) of the residue showed a new signal at 5.00 ppm, corresponding to ArCH_2Br, integration of which indicating the formation of 2-bromomethyl-5-nitro-benzoic acid methyl ester (**8**) with *ca* 75% yield. This benzylic bromide (**8**) was not purified, and the crude residue was dissolved in CH_2Cl_2 (250 cm^3) to be treated with phenol (12 g; 127 mmol), sodium hydroxide (5.08 g; 127 mmol) in water (250 cm^3), and tricaprylmethylammonium chloride (aliquat) (2.50 g; 6.2 mmol) as phase transfer catalyst. The reaction mixture was vigorously stirred at room temperature for 5 h. The organic phase was then separated, washed with water (400 cm^3), dried over $MgSO_4$, filtered, concentrated *in vacuo*, and chromatographed on a column of silica gel (5 x 50 cm) with dichloromethane as eluent. The collected solution (*ca* 600 cm^3) containing all compounds absorbing at 256 nm, was concentrated to *ca* 150 cm^3. Methanol (200 cm^3) was added and the solution concentrated again to *ca* 150 cm^3. Crystallization of the compound (**9**), sparingly soluble in methanol, occurred from the boiling solution as soon as the more volatile dichloromethane was evaporated. The mixture was left at room temperature overnight, the crystals were filtered, abundantly washed with methanol, and air-dried. Analytically pure white crystals of 2-phenoxymethyl-5-nitro benzoic acid methyl ester (**9**) (11.1 g; 60% yield from the starting acid (**6**) were thus obtained, mp = 114°C. ^1H NMR (90 MHz; $CDCl_3$; TMS): 8.93, d (2), 1H (ArH^6); 8.45, dd (2;8), 1H (ArH^4); 8.07, d (8), 1H (ArH^3); 7.2, m, 5H (C_6H_5O); 5.58, s, 2H (ArCH_2O); 4.00, s, 3H ($COOCH_3$). Analysis: calc for $C_{15}H_{13}NO_5$, C: 62.71%; H: 4.56%; N: 4.88%, found C: 62.92%; H: 4.31%; N: 4.98%.

*O_2-m-aB[$CH_2OC_6H_5$]-OH (**10**)*
To a suspension of the methyl ester (**9**) (3.59 g, 12.5 mmol) in 100 cm^3 methanol was added a solution of aq KOH 4 N (12.5 cm^3; 50 mmol). The mixture was stirred at room temperature for 4 h. Water (150 cm^3) was added and methanol was evaporated *in vacuo* at 40°C. The resulting aqueous solution was extracted with CH_2Cl_2 (50 cm^3) and acidified by an excess of aq HCl. The resulting precipitate was dissolved in CH_2Cl_2 (400 cm^3). The organic layer was separated, washed with water (50 cm^3), dried over $MgSO_4$, filtered, and concentrated to *ca* 20 cm^3. The acid (**10**) crystallized from the concentrated solution. The mixture was kept in a freezer overnight, the crystals were filtered, rapidly washed with cold CH_2Cl_2 and air dried, leading to analytically pure 2-phenoxymethyl-5-nitrobenzoic acid (**10**): 2.79 g (82%), mp = 170°C. ^1H NMR (90 MHz; CD_3COCD_3; TMS): 8.93, d (2), 1H (Ar*H*^{6}); 8.55, dd (2; 8), 1H (Ar*H*^{4}); 8.15, d (8), 1H (Ar*H*^{3}); 7.3, m, 5H (C_6H_5O); 5.71, s, 2H (ArCH_2O). Analysis: calc for $C_{14}H_{11}NO_5$, C: 61.54%; H: 4.06%; N: 5.12%, found C: 61.98%; H: 3.84%; N: 5.12%.

*O_2-m-aB[$CH_2OC_6H_5$]-Gly-Gly-Gly-Gly-OEt (**12**)*
2-Phenoxymethyl-5-nitro-benzoic acid (**10**) (2.79 g; 10.2 mmol) was stirred in 25 cm^3 of thionyl chloride and *ca* 0.1 cm^3 of dimethylformamide (DMF), at room temperature for 3 h. The solution was then evaporated under reduced pressure at 40°C. The resulting crude 2-phenoxymethyl-5-nitro-benzoyl chloride (**11**) was dissolved in DMF (40 cm^3) and the solution added drop by drop, at 0°C, to a solution of tetraglycine ethyl ester hydrobromide (3.75 g; 10 mmol) in DMF (50 cm^3) and triethylamine (3 cm^3; 21 mmol). After addition, the reaction mixture was stirred at 0°C for 1 h and at room temperature for 2 h. DMF was evaporated under reduced pressure at 50°C, and the residue was triturated in water (200 cm^3) until a homogeneous solid precipitate was obtained. The solid was filtered, washed successively with H_2O (200 cm^3), 0.5 N aq HCl (200 cm^3), H_2O (100 cm^3), 5% aq $NaHCO_3$ (200 cm^3), H_2O (200 cm^3) and air-dried. It was then triturated in methanol (50 cm^3), filtered, washed with methanol and ether, and dried. Pure peptide (**12**) (3.83 g; 72% yield) was thus obtained as a pale brown powder (mp = 197–200°C), and could be used in the next step without further purification. A sample of 50 mg was dissolved in hot DMF and crystallized at room temperature after addition of aqueous methanol, leading to analytically pure crystals (powder), mp = 199–203°C. ^1H NMR (90 MHz; DMSO–d_6; TMS): 9.09, t (5.6), 1H (N*H* Gly); 8.42, d (2), 1H (Ar*H*^{6}); 8.36, dd (2; 8.6), 1H (Ar*H*^{4}); 8.34, t (masked), 1H (N*H* Gly); 8.28, t (5.8), 1H (N*H* Gly); 8.23, t (5.8), 1H (N*H* Gly); 7.88, d (8.6), 1H (Ar*H*^{3}); 7.3, m, 2H, and 7.0, m, 3H (C_6H_5O); 5.39, s, 2H (ArCH_2O); 4.06, q (7.1), 2H (OCH_2OCH_3); 3.96; d (5.5), 2H (CH_2 Gly); 3.81, d (5.5), 4H (2 superposed CH_2 Gly); 3.75, d (5.6), 2H (CH_2 Gly); 1.17, t (7.1), 3H (OCH_2CH_3). Analysis: calc for $C_{24}H_{27}N_5O_9$, 0.5 H_2O, C: 53.52%; H: 5.24%; N: 13.00%, found C: 53.48%; H: 5.09%; N: 12.50%.

Boc-L-Phe-m-*aB[$CH_2OC_6H_5$]-Gly-Gly-Gly-Gly-OEt (**14**)*
The peptide (**12**) (1.06 g; 2 mmol) was dissolved in hot DMF. Methanol (75 cm^3) and platinum oxide (0.15 g) were added to the warm solution, which was hydrogenated on a Parr apparatus at room temperature for 2 h. Methanol (75 cm^3) was added and the solution was filtered in order to remove the catalyst. Analytical tlc of the filtrate with eluent I (15/85) showed a new unique spot, highly fluorescent on UV light at both 256 nm and 366 nm, corresponding to the aromatic amine (**13**). The compound (**13**) was not isolated and was used directly in the next step, because of its instability in solution. Thus, methanol and DMF were rapidly evaporated *in vacuo* at 40°C. The residue was dissolved in DMF (1 cm^3), and CH_2Cl_2 (25 cm^3) was added. The clear solution was cooled at 0°C, and solutions of Boc-L-phenylalanine (0.795 g; 3 mmol) in CH_2Cl_2 (10 cm^3) and DCC (0.618 g; 3 mmol) in CH_2Cl_2 (15 cm^3) were successively added. The reaction mixture was stirred from 0°C during 2 h to room temperature overnight. The solvents were evaporated, ethyl acetate (300 cm^3) was added, and the dicyclohexylurea precipitate was removed by filtration. The filtrate was extracted successively with 0.5 M aq HCl (2 x 100 cm^3), H_2O (100 cm^3), 5% aq $NaHCO_3$ (2 x 100 cm^3), H_2O (2 x 100 cm^3), dried over $MgSO_4$, filtered and evaporated. The residue was chromatographed on a column of silica gel (3 x 59 cm^3). Elution with eluent system I (10/90) led to the pure peptide (**14**): 1.002 g (67%), as a white solid form, mp = 95–105°C. R_F = 0.30 (I: 10/90); 0.65 (I: 20/80). ^1H NMR (90 MHz; DMSO–d_6): 10.33, s, 1H (N*H* aB); 8.70, m, 1H (N*H* Gly); 8.30, m, 3H (3 N*H* Gly); 7.82, s, 1H (Ar*H*^{6}); 7.79, d (8), 1H (Ar*H*^{4}); 7.54, d (8), 1H (Ar*H*^{3}); 7.35, m, 5H (Ar*H* Phe); 7.29, m, 2H, and 7.02, m, 3H (C_6H_5O); 5.25, s, 2H (ArCH_2O); 4.38, m, 1H (CH_α Phe); 4.11, q (7), 2H (OCH_2CH_3); 3.95, m, 8H (4 CH_2 Gly); 2.98, m, 2H ($CH_2\beta$ Phe); 1.33, s, 9H (CH_3)$_3$C Boc); 1.20, t (7), 3H (OCH_2CH_3). ^1H NMR (90 MHz; CD_3OD): 7.87, s, 1H (Ar*H*^{6}); 7.59, s, 2H (Ar*H*^{3}*H*^{4}); 7.31, m, 5H (Ar*H* Phe); 7.30, m, 2H, and 7.02, m, 3H (C_6H_5O); 5.30, s, 2H (ArCH_2O); 4,50, m, 1H (CH_α Phe); 4.14, q (7), 2H (OCH_2CH_3); 4.12, s, 2H (CH_2 Gly); 3.93, s, 6H (3 CH_2 Gly); 3.07, m, 2H ($CH_2\beta$ Phe); 1.39, s, 9H ([CH_3]$_3$C Boc); 1.17, t (7), 3H (OCH_2CH_3). Optical rotation: $[\alpha]^{25}$ (c 0.7; MeOH) = +21.6° (589); +22.6° (578); +26.4° (546); +52.7° (436); +105.3° (365). Analysis: calc for $C_{38}H_{46}N_6O_{10}$, 0.5 H_2O, C: 60.38%; H: 6.27%; N: 11.12%, found C: 60.38%; H: 6.03%; N: 11.09%.

Boc-L-Phe-m-*aB[$CH_2OC_6H_5$]-Gly-Gly-Gly-Gly-NHNH_2 (**15**)*
The peptide (**14**) (1.95 g; 2.6 mmol) was dissolved in methanol (125 cm^3). A large excess of hydrazine monohydrate (4 cm^3; 82 mmol) was added, and the solution was stirred at room temperature for 24 h. Methanol and the excess of hydrazine were evaporated *in vacuo*. The residue was evacuated at 40°C/0.1 mm for 1 h, solubilized in 20 cm^3 methanol, and precipitated by addition of ether (200 cm^3). The precipitate was filtered, washed with ether and dried, leading to 1.704 g (89%) of peptide (**15**) as a white powder, mp = 120–125°C. R_F = 0.41 (I: 20/80); 0.70 (II). ^1H NMR (90 MHz; CD_3OD; TMS): 7.89, s, 1H (Ar*H*^{6}); 7.60, s, 2H (Ar*H*^{3}*H*^{4}); 7.33, m, 5H (Ar*H* Phe); 7.29, m, 2H, and 7.02, m, 3H (C_6H_5O); 5.29, s, 2H (ArCH_2O); 4.50, m, 1H (CH_α Phe); 4.11, s, 2H (CH_2 Gly); 3.93, s, 4H (2 CH_2 Gly); 3.89, s, 2H (CH_2 Gly); 3.09, m, 2H ($CH_2\beta$ Phe); 1.42, s, 9H ([CH_3]$_3$C Boc). Optical rotation: $[\alpha]^{25}$ (c 0.8; MeOH) = +22.1° (589); +23.4° (578); +27.3° (546); +54.4° (436); +108.2 (365). Analysis: calc for $C_{36}H_{44}N_8O_9$, 0.5 H_2O, C: 58.28%; H: 6.11%; N: 15.10%, found C: 57.95%; H: 5.80%; N: 14.95%.

H-(L)-Phe-m-*aB[$CH_2OC_6H_5$]-Gly-Gly-Gly-Gly-NHNH_2, 2 HCl (**16**)*
The peptide (**15**) (1.920 g; 2.62 mmol) was dissolved in glacial acetic acid (50 cm^3). A solution of *ca* 2 M HCl in acetic acid (50 cm^3) was added, leading to the formation of a precipitate. The mixture was stirred at room temperature for 0.5 h and ether (150 cm^3) was added. After decantation, the organic phase was separated. The precipitate was washed several times with ether, dissolved in methanol (150 cm^3) and evaporated *in vacuo*. The resulting solid residue was dried *in vacuo* in a dessicator over KOH pellets, leading to 1.78 g (96%) of peptide (**16**) as a pale

brown powder, mp = 165–175°C. R_F = 0.04 (I: 2/8); 0.53 (II). ^{1}H NMR (90 MHz; CD_3OD; TMS): 7.91, s, 1H (Ar*H*^{6}); 7.62, s, 2H (Ar*H*^{3}*H*^{4}); 7.39, m, 5H (Ar*H* Phe); 7.28, m, 2H, and 7.02, m, 3H (C_6H_5O); 5.29, s, 2H (ArCH_2O); 4.34, m, 1H (CH_α Phe); 4.11, s, 3.99, s, and 3.94, s, 8H (4 CH_2 Gly); 3.29, m, 2H ($CH_2\beta$ Phe). Optical rotation: $[\alpha]^{25}$ (c 0.7; MeOH) = +51.0° (589); +53.6° (578); +62.3° (546); +122.7° (436); +240.7° (365). Analysis: calc for $C_{31}H_{36}N_8O_7$, 2 HCl, 2 H_2O; C: 50.20%; H: 5.70%; N: 15.11%, found C: 50.15%; H: 5.35%; N: 14.91%.

Cyclo[-(L)-Phe-m-*aB(CH$_2$OC$_6$H$_5$)-Gly-Gly-Gly-Gly-] (**2**)*
The peptide (**16**) (0.282 g; 0.4 mmol) was dissolved in DMF (8 cm^3). After cooling at –40°C, a solution of 8 N HCl in THF (0.400 cm^3; 3.2 mmol), and isoamylnitrite (0.080 cm^3; 0.6 mmol) were successively added. The reaction mixture was stirred at –40°C for 0.5 h. The resulting peptide azide (**17**) was diluted with cold DMF (80 cm^3). The solution was then brought to pH 9 by addition of diisopropylethylamine (0.85 cm^3) and kept in a refrigerator for 24 h. The reaction mixtures from 8 different experiments were combined at this stage (2.11 g; 3 mmol of starting peptide **16** used altogether). 5% aq K_2CO_3 (200 cm^3; 72 mmol) was added, the mixture was concentrated to *ca* 100 cm^3, filtered on a fritted glass, and evaporated to dryness. The residue was dissolved in methanol, and the concentrated solution (*ca* 5 cm^3) was chromatographed on a column of silica gel (3 x 45 cm), with eluent system I (1/9) (100 cm^3) then I (2/8) (500 cm^3). The product containing fractions were pooled and evaporated *in vacuo*. The residue was solubilized in MeOH (30 cm^3)/CH_2Cl_2 (50 cm^3)/H_2O (10 cm^3), dichloromethane and methanol were evaporated at 50°C, and after concentration to *ca* 10 cm^3, crystallization occured at room temperature. The crystals were filtered, abundantly washed with water, and dried; weight was 0.747 g. Two further crystallizations in a similar manner and chromatography of the combined mother liquors followed by crystallization, yielded altogether 0.648 g (36%) of analytical pure cyclopeptide (**2**), obtained as a white powder, mp = 268–272°C. R_F = 0.65 (I: 2/8); 0.75 (II); ninhydrin negative single spot. ^{1}H NMR (500 MHz; DMSO–d_6); see [17]. (300 MHz; CD_3COOD): 8.14, dd (8.4; 2.1), 1H (Ar*H*^{4}); 7.77, d (2.1), 1H (Ar*H*^{6}); 7.66, d (8.4), 1H (Ar*H*^{3}); 7.36, m, 5H (Ar*H* Phe); 7.35, m, 2H, and 7.05, m, 3H (C_6H_5O); 5.32, s, 2H (ArCH_2O); 6.31, dd (4.8; 9.8), 1H (CH_α Phe); 5.69, d (17.1) and 5.49, d (17.2), 2H (CH_2 Gly); 5.69, d (17.1) and 5.62, d (16.9), 2H (CH_2 Gly); 5.65, d (17.1) and 5.48, d (17.0), 2H (CH_2 Gly); 5.46, d (15.9) and 5.26, d (15.9), 2H (CH_2 Gly); 4.87, dd (4.8; 14.2), 1H, and 4.52, dd (9.8; 14.2), 1H ($CH_2\beta$ Phe). Optical rotation: $[\alpha]^{25}$ (c 0.4; DMF) = –61.3° (589); –64.4° (578); –74.8° (546); –141.6° (436); –260.2° (365). Mass spectrum (FAB–): 599 $(M–H)^-$. Analysis: calc for $C_{31}H_{32}N_6O_7$, H_2O, C: 60.18%; H: 5.54%; N: 13.58%, found C: 60.26%; H: 5.54%; N: 13.12%.

Cyclo[-(L)-Phe-m-*aB(CH$_2$Br)-Gly-Gly-Gly-Gly-] (**3**)*
The peptide (**2**) (0.030 g; 0.05 mmol) was dissolved in warm acetic acid (2.55 cm^3). The solution was cooled to room temperature, and a solution of 33% HBr in acetic acid (0.45 cm^3) was added. The reaction mixture was stirred at room temperature for 1 h. Ether (20 cm^3) was added, leading to precipitation of a fine solid. The precipitate was decanted and the supernatant solution discarded. The precipitate was washed several times with ether, collected by centrifugation, and dissolved in 2 cm^3 of eluent I (20/80). The solution was chromatographed on a column of silica gel (1.2 x 20 cm) with eluent I (20/80). The product containing fractions were pooled, and the solution concentrated at 20°C to *ca* 4 cm^3. Ether (75 cm^3) was added, leading to precipitation of a light white solid. The precipitate was collected by centrifugation, and dried *in vacuo*. The bromide (**3**) was obtained as a white powder: yield 0.021 g (71%), mp = 180–200°C (decomp). R_F = 0.60 (I: 20/80); 0.70 (II); ninhydrin negative single spot for fresh MeOH of DMF solutions (several spots corresponding to unidentified decomposition products observed after *ca* 3 h at room temperature). ^{1}H NMR (300 MHz; CD_3OD): 8.15, dd (2.1; 8.5), 1H (Ar*H*); 7.92, d (2.2), 1H (Ar*H*); 7.66, d (8.5), 1H (Ar*H*); 7.47, m, 5H (Ar*H* Phe); 5.00, s, 2H (ArCH_2Br); 4.66, m, 1H (CH_α Phe); 4.2–3.7, m, 8H (4 CH_2 Gly); 3.59, m, 1H, and 3.20, m, 1H ($CH_2\beta$ Phe). (CD_3COOD): 8.03, d (8.3), 1H (Ar*H*^{4}); 7.79, s, 1H (Ar*H*^{6}); 7.56, d (8.5), 1H (Ar*H*^{3}); 7.36, m, 5H (Ar*H* Phe); 4.93, dd (5; 10), 1H (CH_α Phe); 4.86, s, 2H (ArCH_2Br); 4.43, d (16.4), 1H, and 4.35, d (16.4), 1H (CH_2 Gly); 4.34, d (16.5), 1H, and 4.17, d (16.4), 1H (CH_2 Gly); 4.27, d (16.6), 1H, and 4.10, d (16.5), 1H (CH_2 Gly); 4.09, d (16.0), 1H, and 3.92, d (16.1), 1H (CH_2 Gly); 3.47, dd (5; 15), 1H, and 3.17, dd (10; 15), 1H ($CH_2\beta$ Phe). Optical rotation: $[\alpha]^{25}$ (c 0.12; DMF) = –37.5° (589); –40.0° (578); –46.7° (546); –86.7° (436); –160.8° (365). Mass spectrum (FAB–): 586, 588 (M^- for ^{79}Br and ^{81}Br).

Cyclo[-L-Phe-m-*aB(CH$_2$OAc)-Gly-Gly-Gly-Gly-] (**4**)*
In a round bottomed flask containing the bromide (**3**) (0.025 g; 0.04 mmol) and an excess of potassium acetate (0.3 g), was added DMF (3 cm^3). The mixture was stirred at room temperature overnight, and evaporated to dryness *in vacuo*. A solution of MeOH/CH_2Cl_2 (20/80) (25 cm^3) was added, the mixture was stirred for a few minutes, and filtered. The filtrate was evaporated and the residue chromatographed on a column of silica gel (1.2 x 23 cm) with eluent I (20/80). The product containing fractions were pooled, the solution was evaporated, the residue was dissolved in methanol, and precipitated with ether. The precipitate was collected by centrifugation, washed with ether, and dried. The acetate (**4**) was obtained as a white powder: yield 0.018 g (75%), mp = 190–210°C (decomp). R_F = 0.6 (I: 20/80); 0.55 (II); ninhydrin negative single spot. ^{1}H NMR (300 MHz; CD_3OD): 8.20, dd (2.1; 8.4), 1H (Ar*H*^{4}); 7.93, d (2.2), 1H (Ar*H*^{6}); 7.64, d (8.4), 1H (Ar*H*^{3}); 7.47, m, 5H (Ar*H* Phe); 5.49, d (12.9), 1H, and 5.43, d (12.9), 1H (ArCH_2OAc); 4.85, dd (4.6; 10.1), 1H (CH_α Phe); 4.32, d (16.7), 1H, and 4.09, d (16.9), 1H (CH_2 Gly); 4.25, s, 2H (CH_2 Gly); 4.24, d (17.1), 1H, and 4.09, d (16.9), 1H (CH_2 Gly); 4.01, d (15.6), 1H, and 3.82, d (15.7), 1H (CH_2 Gly); 3.60, dd (4.5; 14.0), 1H, and 3.18, dd (10.3; 14.0), 1H ($CH_2\beta$ Phe); 2.25, s, 3H (CH_3COOCH_2Ar). (DMSO–d_6): 9.12, s, 1H (ArN*H*CO); 8.85, m (t like), 1H (N*H* Gly); 8.70, m (t like), 1H (N*H* Gly); 8.64, d (8.3), 1H (N*H* Phe); 8.20, d (8.5), 1H (Ar*H*^{4}); 8.11, m (t like), 1H (N*H* Gly); 7.75, m (t like), 1H (N*H* Gly); 7.73, s, 1H (Ar*H*^{6}); 7.42, d (8.5), 1H (Ar*H*^{3}); 7.26, m, 5H (Ar*H* Phe); 5.23, d (12.5) and 5.17, d (12.5), 2H (ArCH_2OAc); 4.51, m, 1H (CH_α Phe); 4.0–3.5, m, 8H (4 CH_2 Gly); 3.38, m, 1H, and 2.96, m, 1H ($CH_2\beta$ Phe); 2.03, s, 3H (CH_3COOCH_2Ar). Optical rotation: $[\alpha]^{25}$ (c 0.13; DMF) = –30.8° (589); –32.3° (578); –36.9° (546); –70.0° (436); –126.2° (365). Mass spectrum (FAB–): 566 ($M^{\bullet-}$).

Cyclo[-L-Phe-m-*aB(CH$_2$OH)-Gly-Gly-Gly-Gly-] (**18**)*
A solution of the acetate (**4**) (0.066 g; 0.12 mmol) in methanol (18 cm^3) containing triethylamine (2 cm^3) was stirred at room temperature for 48 h. After evaporation, the residue was chromatographed on a column of silica gel (1.3 x 20 cm) with eluent I (20/80). The product containing fractions were pooled, the solution was concentrated to *ca* 2 cm^3, and precipitated by addition of ether. The precipitate was collected by centrifugation, washed several times with ether, and dried *in vacuo* to

yield 0.043 g (70%) of alcohol (**18**) as a white powder, mp = 200–220°C (decomp). R_F = 0.50 (I: 20/80); ninhydrin negative single spot. ^{1}H NMR (300 MHz; CD_3OD): 8.23, dd (2.2; 8.3), 1H (Ar*H*^{4}); 8.00, d (2.2), 1H (Ar*H*^{6}); 7.63, d (8.3), 1H (Ar*H*^{3}); 7.49, m, 5H (Ar*H* Phe); 4.87, s, 2H (ArCH_2CO); 4.85, dd (partly masked; 4.4 only seen), 1H (CH_α Phe); 4.38, d (16.8), 1H, and 4.11, d (16.8), 1H (CH_2 Gly); 4.30, d (16.3), 1H, and 4.23, d (16.3), 1H (CH_2 Gly); 4.28, d (17.1), 1H, and 4.00, d (17.1), 1H (CH_2 Gly); 4.03, d (15.4), 1H, and 3.78, d (15.4), 1H (CH_2 Gly); 3.65, dd (4.3; 14.2), 1H, and 3.17, dd (10.6; 14.2), 1H (CH_2β Phe). Optical rotation: $[\alpha]^{25}$ (c 0.2; DMF) = –65.9° (589); –69.4° (578); –80.0° (546); –152.8° (436); –282.1° (365).

*Cyclo[-L-Phe-*m*-aB(CH$_2$Cl)-Gly-Gly-Gly-Gly-] (**5**)*

The alcohol (**18**) (0.0157 g; 0.03 mmol) was dissolved in DMF (0.5 cm^3), and to the solution stirred at 0°C was added 0.25 cm^3 of a cold, freshly prepared solution of thionyl chloride (0.17 cm^3) in DMF (5 cm^3). The reaction mixture was stirred at 0°C for 1 min, then at room temperature for 3 min, and evaporated to dryness *in vacuo*. This experiment was repeated several times, with altogether 0.0443 g (0.08 mmol) of alcohol (**18**). The combined residue was chromatographed on a column of silica gel (1.3 x 20 cm) with eluent I (15/85). The product containing fractions were pooled, the solution was evaporated *in vacuo* at 20°C, the residue was dissolved in methanol (1 cm^3), and precipitated with ether. The precipitate was collected by centrifugation, washed with ether, and dried, leading to 0.0268 g (58%) of chloride (**5**) as a white powder, mp = 170–230°C (decomp). R_F = 0.70 (I: 20/80); 0.75 (II); ninhydrin negative single spot for fresh MeOH or DMF solutions (unalterated after *ca* 12 h at room temperature). ^{1}H NMR (300 MHz; CD_3OD): 8.17, dd (2.1; 8.4), 1H (Ar*H*); 7.94, d (2.1), 1H (Ar*H*); 7.68, d (8.4), 1H (Ar*H*); 7.48, m, 5H (Ar*H* Phe); 4.86, dd (4.7, 10.0), 1H (CH_α Phe); 4.83, s 2H (ArCH_2Cl); 4.32, d (17.0), 1H and 4.12, d (17.0), 1H (CH_2 Gly); 4.29, s, 2H (CH_2 Gly); 4.23, d (16.9), 1H and 4.09, d (16.8), 1H (CH_2 Gly); 4.01, d (15.8), 1H and 3.85, d (15.8), 1H (CH_2 Gly); 3.59, dd (4.7; 14.0), 1H and 3.19, dd (10.1; 14.0), 1H (CH_2β Phe). Optical rotation: $[\alpha]^{25}$ (c 0.8; MeOH) = –23.5° (589); –25.9° (578); –31.8° (546); –64.7° (436); –129.4° (365). Mass spectrum (DCI): 542, 544 (M$^{\bullet-}$ for ^{35}Cl and ^{37}Cl).

Enzymes and substrates

α-Chymotrypsin (3 times crystallized) was obtained from Cooper Biochemical and was shown to have 96% of theoretical activity by active-site titration with PNA [21]. Porcine pancreatic elastase, bovine trypsin and human urokinase were purchased from Serva, Sigma and Choay, respectively. Active-site titrations were carried out with NPGB for trypsin [22], and with Z-Ala-Ala-Pro-azaAla-Ala-ONp for elastase [23]. Stock enzyme solutions in 1 mM HCl were prepared shortly before use. The chromogenic substrates Ac-Tyr-pNA for chymotrypsin, Suc-Ala$_3$-pNA for elastase and Bz-Arg-pNA for trypsin, were purchased from Serva, Sigma and Boehringer, respectively. All solvents and intermediates were of the best available commercial grade. PNA was purchased from Sigma and Z-Ala-Ala-Pro-azaAla-Ala-ONp was a kind gift from J Bieth (université Louis Pasteur de Strasbourg, Illkirch, France).

Enzyme assays

The hydrolytic activities of native α-chymotrypsin, trypsin and elastase, and of their derivatives were examined at 25°C towards the appropriate substrate using a Lambda 5 Perkin–Elmer spectrophotometer equipped with a thermostated cell holder. The pH was measured before and after each run, using a pH-stat Radiometer TTT1c equipped with a type B glass electrode. The assay volume was 1 ml in the following buffers: 0.025 M sodium phosphate, pH 7.5, 0.1 M KCl, for α-chymotrypsin; 0.1 M Tris, pH 8.0 for porcine pancreatic elastase; 0.05 M Tris, pH 7.5, 0.01 M $CaCl_2$, for bovine trypsin and 0.05 M Tris, pH 7.4, 0.04 M NaCl, 0.01% (v/v) Tween 80 for urokinase. The enzyme concentration was usually 200 nM (α-chymotrypsin), 160 nM (trypsin), 250 nM (urokinase) and 50 nM (elastase); the substrate concentrations were usually 100 μM.

Enzyme inactivation

Inactivation experiments were performed at 25°C by following the general protocol described below for α-chymotrypsin.

At time zero, the reagents **2**, **3**, **4**, **5** in solution in dimethylformamide (final concentrations 55–570 μM; 5–6 different concentrations of inhibitor used) were added to a solution of α-chymotrypsin (4.3 μM for **3** and 10 μM for **2**, **4** and **5**) in a 0.025 M sodium phosphate buffer, pH 7.5, 0.1 M KCl. The final concentration of dimethylformamide was 3% (v/v). Aliquots (20 μl) were removed at various time intervals and the reaction was stopped either by simple dilution or by dilution followed by filtration–centrifugation at 4°C on Centricon 10 microconcentrators (Amicon, Danvers, MA) and washing with the buffer. The samples were assayed for remaining enzymatic activity by addition to 1 ml of an appropriate standard assay mixture (see above). The enzyme activity is expressed relative to a chymotrypsin control which was prepared by adding 3 μl of dimethylformamide to 100 μl of a 4.3 or 10 μM α-chymotrypsin solution in buffer, and incubated in parallel to the inactivation experiments. Aliquots were treated in analogous experimental conditions. The apparent pseudo-first-order rate constants (k_{obs}) were obtained from least-squares analysis of semilog plots of the percentage of remaining activity against time. The apparent binding constant K_I and the first-order inactivation rate constant k_{inact} for the reaction of **5** with α-chymotrypsin were then obtained by a Wilkinson type analysis [25] from a double reciprocal plot of k_{obs} *versus* the inhibitor concentration, according to Kitz and Wilson [20].

Assays for inactivation of bovine trypsin, human urokinase and porcine pancreatic elastase by reagents **3** and **5** were carried out by adding an aliquot of **3** and **5** in dimethylformamide (final concentration 100–500 μM) to the enzyme solution in 0.05 M Tris (pH 7.4), 0.04 M NaCl, 0.01% (v/v) Tween 80, 0.05 M Tris (pH 7.5), 0.01 M $CaCl_2$ and 0.1 M Tris (pH 8.0) buffers, for urokinase (0.54 μM), trypsin (8 μM) and elastase (2 μM), respectively. Aliquots (20 μl) were withdrawn at various intervals, and the residual enzymatic activity was measured in the appropriate standard assay mixture.

*Chymotrypsin hydrolysis of cyclopeptides **2** and **4***

Chymotrypsin activity towards cyclopeptides **2** and **4** was measured spectrophotometrically in 0.025 M sodium phosphate buffer, pH 7.5, 0.1 M KCl, 1.5% (v/v) DMSO (**2**) or 1.5% (v/v) DMF (**4**), at 37°C. Cyclopeptide concentration varied from 75–434 μM (**2**) and 100–250 μM (**4**); the enzyme concentration was 1.46 μM (**2**) or 0.83 μM (**4**). The kinetic parameters for the catalyzed hydrolysis of the cyclopeptide **2** were determined by a Lineweaver plot using the treatment of Wilkinson [25]. For the cyclopeptide **4**, the second-order rate constant k_{cat}/K_M was obtained by plotting the initial rates v_i

versus the substrate concentrations, and dividing the slope by the enzyme concentration. The correlation coefficient was greater than 0.99. For these kinetic determinations, the molar absorption coefficients were 1150 $M^{-1}cm^{-1}$ at 310 nm (**2**) and 1012 $M^{-1}cm^{-1}$ at 320 nm (**4**).

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