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Original paper

Synthesis and inhibiting activity of peptidylketones as substrate analogues of papain

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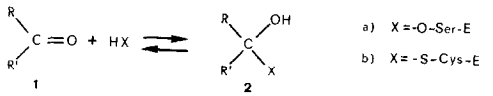
Summary — Four representative peptidylketones were synthesized and evaluated as models of cysteine–protease inhibitors. They were shown to be slow-binding, reversible inhibitors of papain with K_i ranging from 8.4–260 μM. The effectiveness of the inhibitor was enhanced by the introduction of 3 chlorine atoms α to the keto group while a small decrease was observed with analogous substitution by fluorine. The structure–activity relationships are discussed.

Résumé — Synthèse et activité inhibitrice de peptidylcétones comme analogues du substrat de la papaine. *Quatre peptidylcétones représentatives ont été synthetisées et étudiées comme modèles d'inhibiteurs de cysteine–proteases. Elles apparaissent être des inhibiteurs réversibles, à fixation lente, de la papaine avec des K_i de 8.4–260 μM. L'efficacité de l'inhibiteur est augmentée par l'introduction de 3 atomes de chlore en α du groupement carbonyle, alors qu'une faible diminution est observée avec une substitution analogue par le fluor. Les relations structure–activité sont discutées.*

peptidylketones / substrate analogues / papain inhibitors / slow binding

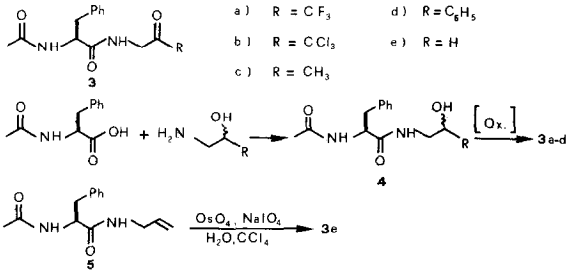
Introduction

Naturally occurring peptidylaldehydes (**1**, R′=H) have been recognized as potent inhibitors of serine proteases by Umezawa [1]. Several synthetic aldehydes structurally related to the acyl portion of peptide substrates proved to be powerful inhibitors of both serine and cysteine proteases. Inhibition was attributed to the exceptional stability of hemiacetals (**2a**,R′=H) or hemithioacetals (**2b**,R′=H) formed between the enzyme active site serine or cysteine and the inhibitor aldehyde carbonyl group (Scheme 1) [2–5].



Scheme 1.

Experimental evidence for covalent tetrahedral complex formation has been obtained for both papain [6] and for serine proteases [7], chiefly by ^{13}C NMR spectroscopy. More recently several powerful inhibitors of hydrolytic enzymes have been synthesized by replacing the aldehyde group of appropriate substrate analogues with a fluoromethylketone moiety. Among them, inhibitors of serine proteases are well represented [8–11], while no example for cysteine proteases is provided in spite of the fact that thiol proteases are implicated in several pathological processes [12]. The present study was undertaken to verify the effectiveness of a trifluoromethylketone as inhibitor of a cysteine protease as papain and to compare its activity with that of similar substrate analogues. To convey to the potential inhibitors those structural features responsible for enzyme–substrate recognition and binding, *N*-acetyl-Phe was chosen as the peptidyl moiety in view of the strong preference of papain for substrates with Phe at P_2 [13]. The *N*′-acetyl-L-phenylalanyl derivatives **3a–3d** (Scheme 2) and the highly active peptidylaldehyde **3e** have been therefore synthesized and tested as inhibitors in papain catalysed hydrolysis of *N*-carbobenzyloxyglycine *p*-nitrophenyl-ester.



Scheme 2.

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Chemistry

The ketone derivatives **3a–3d** were synthesized according to the scheme of Abeles [14]. The intermediate alcohols **4a–4d** were obtained by coupling the appropriate amino-alcohols with *N*-acetyl-L-phenylalanine by using a standard mixed-anhydride method [15]. The mixtures of diastereoisomers, purified by column chromatography on silica gel and by crystallization, were directly submitted to oxidation. The trifluoromethylalcohol **4a** could be cleanly oxidized by the method of Abeles [14] with alkaline permanganate. These conditions, however, proved to be unsuccessful for the preparation of the trichloromethylketone **3b** due to its poor stability in basic media: in fact only the product of basic cleavage, *N*-acetyl-L-phenylalanylglycine, was obtained from the reaction mixture. Known procedures for the oxidation of trichloromethylcarbinols use conditions which are hydrolytic for peptide bonds [16]. The alcohol **4b** proved to be resistant to oxidation with pyridinium chlorochromate in dichloromethane, Jones reagent, acid permanganate, ruthenium tetroxide in carbon tetrachloride–water, or decomposed during the treatment with pyridine chromium trioxide in dichloromethane, with dimethylsulfoxide–acetic anhydride and with alkaline permanganate. However, the trichloromethylalcohol **4b** was easily oxidized in almost quantitative yield by sodium bichromate, in acetic acid at 25°C. The other peptidylalcohols **4c** and **4d** were similarly oxidized by the same method with complete retention of the configuration of the L-phenylalanine moiety. The peptidylaldehyde **3e** [2] was obtained in very high yield, as the only product, by osmium tetroxide–sodium periodate [17] oxidation of *N*-acetyl-L-phenylalanyl-allylamide **5**. The method is reminiscent of the synthesis of α aminoacylaldehydes by periodate oxidation of the corresponding diols [4].

The proposed structures of all new compounds are in accordance with the spectroscopic data (mass, IR, ^{1}H, ^{13}C, and ^{19}F NMR).

Results

Compounds **3a–3d** and **4a–4d** were tested as inhibitors of papain. Reaction progress curves show that inhibition of papain is time-dependent as in Fig. 1, for inhibition by **3b**. The progress curves show that a steady-state velocity is reached slowly and the final velocities are dependent on inhibitor concentration. These results indicate that the decrease in activity was not due to irreversible enzyme inactivation or substrate depletion but to slow-binding inhibition, *i.e.* to a slow onset of activity loss relative to the rate of diffusion [18, 19].

Three kinetically distinct mechanisms have been suggested to describe slow progress of inhibition [19–21]. The first one assumes a slow interaction between enzyme and inhibitor, the second a rapid formation of a preliminary EI′ complex followed by a slow modification to a more stable EI complex and the third one a slow isomerization of the enzyme followed by rapid binding of the inhibitor. These 3 mechanisms are kinetically differentiable if

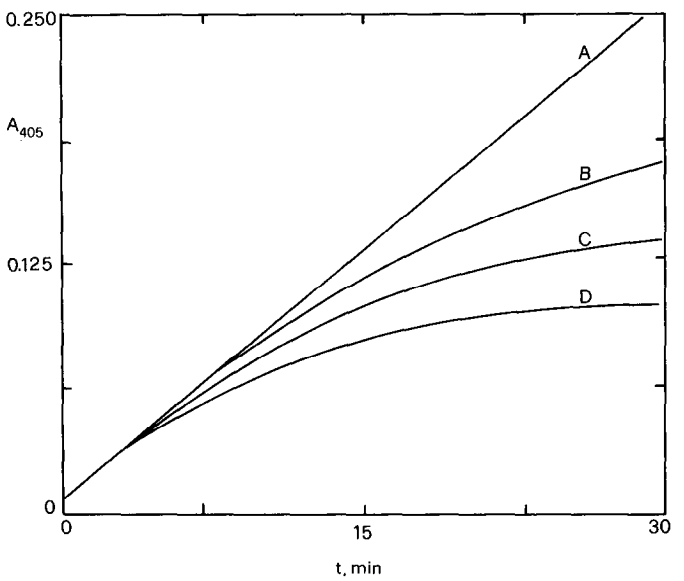


Fig. 1. Progress curves for inhibition of papain by **3b**. Reaction conditions: [E]=6 × 10^{-9} M, [ZGlyONp]=125 μM, 100 mM phosphate buffer pH 6.8, 4 mM EDTA, CH_3CN %=12 (v/v), 25°C. Inhibitor concentrations are 0, 16, 47 and 62 μM for curves **A, B, C, D,** respectively. Reactions were initiated by addition of enzyme.

these conditions are satisfied [20]: i) concentration of substrate is not depleted during the reaction; ii) inhibitor concentration is not changed upon formation of the enzyme–inhibitor complex; iii) the decrease in velocity is represented by:

$$V=V_s+(V_o-V_s)\cdot e^{-k_{obs}t} \quad (1)$$

where V_s, V_o and V are the steady-state, initial and time t velocities, respectively, and k_{obs} is the apparent first order-rate constant for the onset of the steady-state equilibrium. From Equation 1 it follows that a plot of ln $[(V-V_s)/(V_o-V_s)]$ *vs* time will be linear with a slope equal to $-k_{obs}$, as in Fig. 2 for papain inhibition by **3b**. For the first mechanism the following scheme [22] and Equations (2) and (3) are valid.

$$E + S \rightleftharpoons ES \xrightarrow{k_2} ES' \xrightarrow{k_3} E + P_2 \quad (+P_1)$$

$$+I \quad k_{on} \downarrow\uparrow k_{off}$$

$$EI$$

$$K_i=k_{off}/k_{on} \quad (2)$$

$$k_{obs}=k_{on}[I]\,(1+[S]/K_m)+k_{off} \quad (3)$$

According to this simple mechanism the observed rate constant for the approach to steady-state, k_{obs}, is given by Eq. (3) and predicts that a plot of k_{obs} *vs* [I] $(K_m/K_m+[S])$ will be linear with slope and intercept of k_{on} and k_{off}, respectively. Figure 3 shows such a plot for papain inhibition by compound **3b**. All the other inhibitors tested followed the first mechanism. As a matter of fact the plots of k_{obs} *vs*

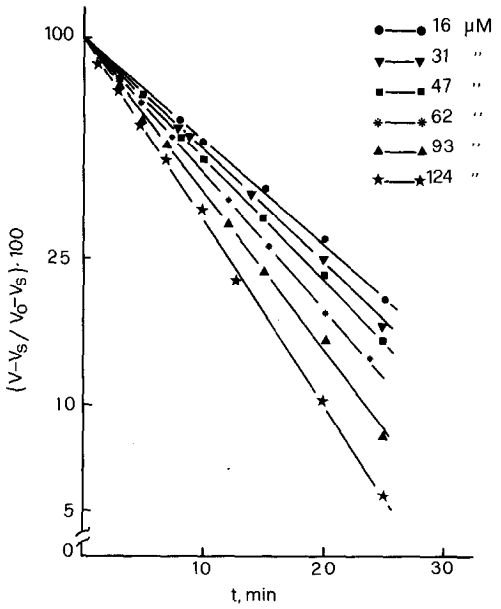


Fig. 2. Determination of k_{obs} for the time-dependent inhibition of papain by the reported concentrations of **3b**. Velocities at various times were measured from data of progress curves (see enzyme assay) and plotted according to Eq. 1 (see text). k_{obs} was determined from the slope of the lines by linear regression analysis.

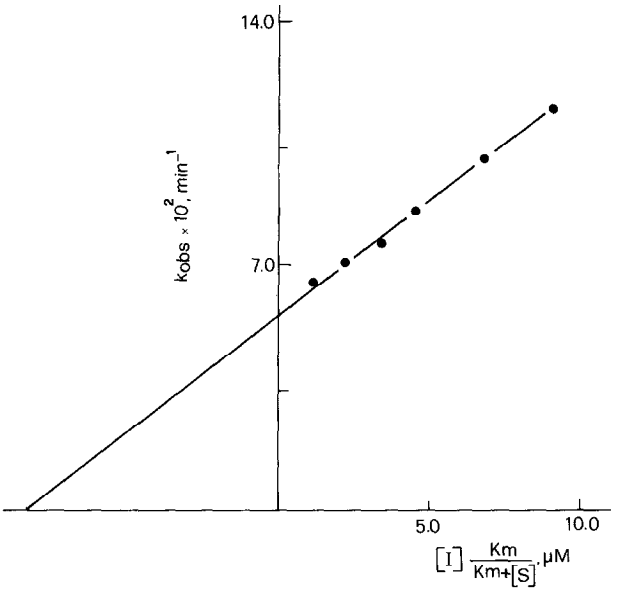


Fig. 3. Kinetics of papain inhibition by **3b** plotted according to Eq. 2 (see text). First-order rate constants for the approach to steady-state are a linear function of [I]. k_{off} and k_{on} values were determined by linear regression analysis of the data.

[I] (K_m / K_m+[S]) give a straight line (Fig. 3) and the initial velocities are all identical and equal the initial velocity in absence of inhibitor (Fig. 1).

If an intermediate EI′ complex did form prior to the formation of EI, k_{obs} would be a hyperbolic function of [I] and the initial velocities of Figure 1 would decrease with increasing [I] [19]. However, this does not exclude the formation of a not detectable, under the conditions used, preassociation complex prior to the step of k_{on}. Table I summarizes the kinetic parameters for the ketones we tested. The most effective is the trichloromethylketone, the others varying within one order of magnitude.

Table I. Inhibition of papain by ketones and alcohols derived from *N*-acetyl-Phe*.

Compound	Range (μM)	k_{on} ($M^{-1} \times min^{-1}$)	k_{off} (min^{-1})	K_i (μM)
3b	15: 124	6550	0.055	8.4
3c	15: 250	4860	0.064	13.2
3d	125: 1000	1280	0.055	43.0
3a	500: 2000	344	0.055	160
4b	1000: 3000	--	--	n.i.**
4c	312: 5000	211	0.055	260
4d	1000: 5000	--	--	n.i.
4a	1000: 5000	--	--	n.i.
Chloral	1000:10000	--	--	n.i.
Trichloroacetone	10000:50000	--	--	n.i.

*100 mM phosphate buffer, pH 6.8; CH_3CN = 12% (v/v); [E]=6×10^{-9} M.
[S] = 125 μM (K_m × 12.5). Replicate determinations indicate standard deviations for the kinetic parameters less than 20%.
**Not inhibitory in the reported range.

Discussion

The ketonic derivatives **3a**–**3d** of *N*-acetyl-L-phenylalanine proved to be (Table I) slow-binding, competitive and reversible papain inhibitors more effective than the corresponding alcohol analogues **4a**–**4d**. They are therefore assumed to be substrate analogues forming tetrahedral complexes [6] with the cysteine 25 SH of the papain (**2b**). The peptidylalcohol **4c**, however, exhibits an unexpected affinity for the enzyme. The stability of its non-covalent complex, which is only ≈ 20-fold lower (10-fold if referred to only one steroisomer) than that of the corresponding ketonic analogue **3c**, may be accounted for by assuming that **4c** behaves as a transition state analogue mimicking the tetrahedral intermediate during the enzymatic amide bond hydrolysis. Transition state analogues of this class are, to our knowledge, unprecedented in the domain of both cysteine and serine proteases while they are well known as renin inhibitors containing statine or statine analogues [23].

The inhibitory activity of the substrate analogues reported in Table I can be discussed in terms of stability of the corresponding hemithioacetals **2b**. It is well known that the tendency of carbonyl compounds to undergo addition of thiols and other nucleophiles is enhanced if the attached groups are small and contain strongly electro-negative atoms [24, 25].

According to the K_i the most stable covalent adduct (K_i=0.046 μM) [2] is formed by the peptidylaldeheyde **3e**. The corresponding methylketone **3c** is some 300-fold less tightly bound. The value of this ratio appears to be in good agreement with the findings of Jencks [24] that equilibrium constants for addition of thiols to acetone are at least 100-fold smaller than for the addition to acetaldehyde. Steric factors appear to play a more important role than electronegativity in determining the stability of the hemithioacetals **2** since both fluorinated and chlorinated derivatives **3a** and **3b** are worse inhibitors than the aldehyde **3e**.

As expected, the trifluoromethylketone **3a** was isolated as the hydrate and proved to be stable in this form. ^{19}F NMR spectra gave well distinguished peaks for the ketone and its hydrate [9]. Careful integration of the signals showed that the hydrate is in equilibrium with 16% of the ketonic form as solution in DMSO and < 1% (undetectable signal) in D_2O. In the reaction of the aliphatic aldehydes with thiols, Jencks [24] found that the stability of hemithioacetals paralleled that of the hydrated forms. Another study on this subject [26] confirms that the main factor leading to the stabilization of hemithioacetals is the presence of an electron-withdrawing group in the α position. Accordingly the R′ groups considered in this paper should stabilize the hemithioacetal **2b** in the order CF_3>CCl_3>Ph>CH_3.

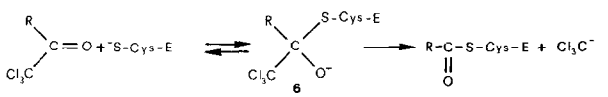
Contrary to these indications, the trifluoromethyl derivative **3a** proved to be the least effective ketonic inhibitor. This finding may be explained taking into account that the equilibrium constants of reactions of emithioacetals formation are referred to CH_2Cl_2 solutions of anhydrous reactants, where the thiol is the only nucleophile present. An interesting observation reported by Field [26] is that no reaction was apparent when hexafluoroacetone trihydrate was employed and when the thiol had to compete with water as an alternative nucleophile. This fact demonstrates the high stability of such hydrates, indicating that the presence of electro-negative groups enhances the affinity of the carbonyl toward hard nucleophiles such as hydroxy groups of the medium or of a catalytic serine rather than for the soft thiol group of the papain cysteine. Unlike the trifluoroanalogue **3a**, the trichloromethylketone **3b** could be isolated in the ketonic form and failed to show any tendency to form hydrates. Furthermore, it proved to be the best papain inhibitor among the peptidylketones tested.

The simultaneous presence of the trichloromethylketo group and of the recognising peptidyl unit is required for the biological activity. In fact chloral, which forms relatively stable hemithioacetals [26], exerts a weak inhibition on papain > 50 mM (≈ 6000-fold the K_i for **3b**) under the same conditions as for the peptidylketone.

In addition 1,1,1-trichloroacetone [27] and the trichloroalcohol **4b** are completely inert in the range of concentrations used. These circumstances strongly suggest that the trichloromethylketone **3b** also behaves as a substrate analogue in spite of the bulkiness of the trichloromethyl group. Unlike trichloromethylketones, monochloromethylketones are well known as irreversible inhibitors of both cysteine and serine acylases: they have been shown [28] to alkylate specifically the catalytic thiol group of papain. The trichloromethylketone **3b**, on the contrary, proved to be a competitive and reversible papain inhibitor and this behaviour is in accordance with the lack of alkylating properties of the trichloromethyl group.

Trichloromethylketones [29], on the other hand, are known as effective acylating reagents. The Cl_3C^- anion is in fact a good leaving group and its release could follow formation of the hemithioketal anion **6**, giving rise to the acylated enzyme. Our kinetic experiments suggest that the rate of formation of acyl-enzyme following the release of Cl_3C^-, if any, is equal or slower than the measured k_{off} for compound **3b** (0.055 min) and thus at least 4 orders of magnitude slower than any turnover rate for papain catalysed hydrolysis.

The stability of the proposed intermediate **5** (Scheme 3) is most likely due to favourable hydrogen bonds and electrostatic interactions of the oxyanion with residues in the oxyanion hole of the enzyme [30]. Furthermore, the physicochemical properties of the Cl_3C^- leaving group should exclude that its abstraction might be catalytically assisted by the enzyme in the same way as the removal of an amino group during the hydrolysis of a natural substrate.



Scheme 3.

In conclusion, the effectiveness of peptidylketones as reversible inhibitors of cysteine proteases is moderately enhanced by the introduction of 3 chlorine atoms α to the keto group. Analogous substitution by fluorine, on the contrary, gives rise to a small decrease of the activity. As

a consequence, trifluoromethylketones with highly enhanced activity against serine proteases, are expected to behave as selective inhibitors without serious interference with cysteine proteases.

Experimental protocols

Melting points (Büchi oil bath apparatus) are uncorrected. Spectra were obtained as follows: IR spectra (for $CHCl_3$ solutions) on a Perkin–Elmer 521 spectrophotometer, EI mass spectra on a Hewlett–Packard 5980 A spectrometer; ^1H NMR spectra on a Varian EM 390 spectrometer; ^{13}C NMR spectra on a Varian XL300 spectrometer operating at 75.43 MHz (the reported assignments are relative to TMS as an internal standard); ^{19}F NMR spectra on a Varian XL 300 spectrometer operating at 282.20 MHz (the reported assignments are relative to $CFCl_3$ as an internal standard). $[\alpha]_D$ were determined with a Schmidt–Haensch 1604 polarimeter. Elemental analyses were performed by Servizio Microanalisi of CNR Area della Ricerca di Roma and were within ± 0.4% of the theoretical values.

Mixed-anhydride coupling. General procedure for the preparation of peptidylalcohols ***4a–4d***

The required aminoalcohols were prepared by known procedures (1-amino-2-propanol [31], 3,3,3-trichloro-1-amino-2-propanol [32] and 3,3,3-trifluoro-1-amino-2-propanol [33]) or supplied by Aldrich (2-amino-1-phenyl-ethanol). *N*-Acetyl-L-phenylalanine (1 mmol) was dissolved in dry THF (1.5 ml) and anhydrous *N*-methylmorpholine (1 mmol) was added. The solution was cooled to −15°C, *i*-butyl-chloroformate (1 mmol) was added and the solution was stirred for 30 min. The appropriate aminoalcohol (1 mmol) was dissolved in THF (1 ml) and added while a temperature of −15°C was maintained. The resulting reaction mixture was stored for 15 h at 4°C in a refrigerator, allowed to warm at room temperature and filtered, after which the solvent was removed at reduced pressure. The residue was dissolved in EtOAc, washed with 1 N HCl, saturated $NaHCO_3$ and brine. After drying over Na_2SO_4 the solvent was removed under reduced pressure to give the crude product which was purified by chromatography on silica gel.

N-*Acetyl-L-phenylalanyl-1-amino-3,3,3-trifluoropropan-2-ol* ***4a***

Crystallization from 1,2-dichloroethane gave a white solid: yield 90%; mp 137–138°C; IR: main peaks at 3429, 3327, 2956, 1729, 1665, 1600, 1511, 1373, 1274 cm^{-1}. ^1H NMR (d_6–DMSO) δ 1.76 (s, 3, CH_3CON), 2.6–3.3 (m, 2, $PheCH_2$), 2.6–3.3 (m, 1, C*H*OH), 4.00 (m, 2, NCH_2), 4.53 (m, 1, PheCH), 6.40 (dd, 1 CHO*H*, mixture of diastereoisomers), 7.30 (s, 5, $PheC_6H_5$), 8.15 (d, 1, *J*=9 Hz, AcNH), 8.30 (t, 1, *J*=5.5 Hz, PheCONH). Anal. calcd. for $C_{14}H_{17}F_3N_2O_3$; C, 52.83; H, 5.38; N, 8.80; found: C, 52.70; H, 5.50; N, 8.63.

N-*Acetyl-L-phenylalanyl-1-amino-3,3,3-trichloropropan-2-ol* ***4b***

Crystallization from 1,2-dichloroethane gave a white solid: yield 78%; mp 122–123°C; IR: main peaks at 3435, 3327, 2927, 1706, 1605, 1501, 1453, 1373, 1302 cm^{-1}. ^1H NMR (d_6–DMSO) δ 1.77 (s, 3, CH_3CON), 2.6–3.3 (m, 2, $PheCH_2$), 2.6–3.3 (m, 1, C*H*OH), 4.00 (m, 2, NCH_2), 4.60 (m, 1, PheCH), 6.47 (dd, 1, CHO*H*, mixture of diastereoisomers), 7.30 (s, 5, $PheC_6H_5$), 8.15 (d, 1, *J*=9 Hz, AcNH), 8.33 (t, 1, *J*=5.5 Hz, PheCONH). Anal. calcd. for $C_{14}H_{17}Cl_3N_2O_3$: C, 45.92; H, 4.64; N, 7.65; Cl, 28.66; found: C, 45.83; H, 4.67; N, 7.31; Cl, 28.97.

N-*Acetyl-L-phenylalanyl-1-aminopropan-2-ol* ***4c***

Crystallization from EtOAc gave a white solid: yield 72%; mp 109–110°C; IR: main peaks at 3427, 3327, 2994, 1659, 1602, 1530, 1512, 1453, 1375, 1289 cm^{-1}. ^1H NMR (d_6–DMSO) δ 0.97 (dd, 3, CH_3CH, mixture of diastereoisomers), 1.75 (s, 3, CH_3CON), 3.00 (m, 2, $PheCH_2$), 3.00 (m, 2, NCH_2), 3.60 (m, 1, C*H*OH), 4.50 (m, 1, PheCH), 4.57 and 4.63 (s, 1, CHO*H*, mixture of diastereoisomers), 7.28 (s, 5, $PheC_6H_5$), 7.92 (t, 1, *J*=5 Hz, PheCONH), 8.13 (d, 1, *J*=9 Hz, AcNH). Anal. calcd. for $C_{14}H_{20}N_2O_3$: C, 63,58; H, 7.57; N, 10.59; found: C, 63.47; H, 7.55; N, 10.50.

N-*Acetyl-L-phenylalanyl-2-amino-1-phenylethanol* ***4d***

Crystallization from EtOAc gave a white solid: yield 80%; mp 143–144°C; IR: main peaks at 3429, 3327, 2996, 1659, 1602, 1529, 1496, 1452, 1372, 1289 cm^{-1}. ^1H NMR (d_6–DMSO) δ 1.76 (s, 3, CH_3CON), 2.85 (bs, 2, $PheCH_2$), 3.30 (bs, 2, NCH_2), 4.60 (bs, 1, PheCH), 4.60 (bs, 1, C*H*OH), 5.45 (bs, 1, CHO*H*), 7.28 (s, 5, $PheC_6H_5$), 7.37 (s, 5, C_6H_5), 8.10 (bs, 1, AcNH), 8.10 (bs, 1, PheCONH). Anal. calcd. for $C_{19}H_{22}N_2O_3$: C, 69.93; H, 6.74; N, 8.58; found: C, 70.21; H, 6.79; N, 8.42.

N-*Acetyl-L-phenylalanyl-1-amino-3,3,3-trifluoropropan-2-one* ***3a***

To a solution of the trifluoroalcohol **4a** (200 mg, 0.628 mmol) in 0.3 N NaOH (5 ml), $KMnO_4$ (132 mg, 0.837 mmol) in 0.3 N NaOH (10 ml) was added drop-wise under stirring. After 30 min the permanganate excess was destroyed by careful addition of 40% formaldehyde and the mixture was immediately extracted with EtOAc. The organic layer was washed with brine, dried over Na_2SO_4 and evaporated at reduced pressure to give a white solid residue (170 mg). Careful crystallization from anhydrous Et_2O / *n*-hexane gave the pure trifluoromethylketone **3a** as a white solid (80 mg, 40%): mp 105–107°C; $[\alpha]_D^{20}$ 8° (c=1.0, MeOH); IR: main peaks at 3682, 3431, 2930, 1659, 1601, 1496, 1177 cm^{-1}; ^1H NMR (d_6–DMSO) δ 1.93 (s, 3, CH_3CON), 2.90 (m, 2, $PheCH_2$), 3.44 (d, 2, *J*=5.5 Hz, NCH_2), 4.60 (m, 1, PheCH), 7.12 (s, 2, $C(OH)_2$), 7.30 (s, 5, $PheC_6H_5$), 8.03 (t, 1, *J*=5.5 Hz, PheCONH), 8.17 (d, 1, *J*=9 Hz, AcNH); ^{13}C NMR (d_6–DMSO) δ 22.3 (CH_3CON), 37.3 ($PheCH_2$), 43.2 (NCH_2), 53.8 (PheCH), 91.8 (J_{CF}=31 Hz, $C(OH)_2$), 123.6 (J_{cf}=276 Hz, CF_3), 126–138 ($PheC_6H_5$), 169.1 (PheCONH), 172.5 (CH_3CON). ^{19}F NMR (d_6–DMSO) δ −108.1, s (ketone form); −94.8, s (hydrated form). The ketone to hydrate ratio was 16:84 by integration; ^{19}F NMR (D_2O) δ −92.9, s; the ^{19}F signal of the ketone form could not be observed in D_2O solution. Mass spectrum, *m/e* M^+ 316. Anal. calcd. for $C_{14}H_{17}F_3N_2O_4$: C, 50.30; H, 5.13; N, 8.38; found: C, 50.69; H, 5.25; N, 8.46.

General procedure for the preparation of compounds ***3b–3d***

A solution prepared by dissolving $Na_2Cr_2O_7.2H_2O$ (0.5 mmol) and H_2SO_4 (2 mmol) in AcOH (2.5 ml) was added dropwise, under stirring to the appropriate peptidylalcohol (1 mmol) in AcOH (2 ml). After 10 min at room temperature the reaction was quenched by addition of iPrOH (0.3 ml) and further stirred for 10 min. The reaction mixture was then poured in EtOAc (75 ml) and the solution sequentially washed with brine, $NaHCO_3$ saturated solution and brine. After drying over Na_2SO_4 the solvent was removed at reduced pressure to give the crude product.

N-*Acetyl-L-phenylalanyl-1-amino-3,3,3-trichloropropan-2-one* ***3b***

The crude material was triturated with Et_2O to give a white solid: yield 83%; mp 50–52°C; $[\alpha]_D^{20}$ = −6° (c=1.0, MeOH); IR: main peaks at 3669, 3424, 3299, 2995, 1765, 1660, 1605, 1505, 1373, 1251 cm^{-1}. ^1H NMR ($CDCl_3$) δ 1.92 (s, 3, CH_3CON), 3.10 (m, 2, $PheCH_2$), 4.80 (d, 2, *J*=5 Hz, NCH_2), 4.97 (m, 1, PheCH), 7.15 (d, 1, *J*=8 Hz, AcNH), 7.25 (s, 5, $PheC_6H_5$), 7.80 (t, 1, *J*=5 Hz, PheCONH); ^{13}C NMR ($CDCl_3$) δ 22.6 (CH_3CON), 38.0 ($PheCH_2$), 41.8 (NCH_2), 54.2 (PheCH), 93.8 (CCl_3), 127–136 ($PheC_6H_5$), 170.6 (PheCONH), 172.1 (CH_3CON), 186.5 (CH_2CO). Anal. calcd. for $C_{14}H_{15}Cl_3N_2O_3$; C, 45.96; H, 4.10; Cl, 29.13; N, 7.66; found: C, 45.93; H, 4.31; Cl, 29.01; N, 7.47.

N-*Acetyl-L-phenylalanyl-1-aminopropan-2-one* ***3c***

Chromatography on silica gel followed by crystallization from benzene gave a white solid: yield 73%; mp 104–105°C $[\alpha]_D^{20}$ −3° (c=1.0, MeOH); IR: main peaks at 3419, 3316, 2996, 1729, 1660, 1605, 1512, 1434, 1372 cm^{-1}. ^1H NMR ($CDCl_3$) δ 1.92 (s, 3, CH_3CON), 2.08 (s, 3, CH_3COC), 3.10 (m, 2, $PheCH_2$), 4.05 (d, 2, *J*=5 Hz, NCH_2), 4.90 (m, 1, PheCH), 6.95 (d, 1, *J*=8 Hz, AcNH), 7.22 (t, 1, *J*=5 Hz, PheCONH), 7.26 (s, 5, $PheC_6H_5$); ^{13}C ($CDCl_3$) δ 23.0 (CH_3CON), 27.2 (CH_3COC), 38.4 ($PheCH_2$), 49.7 (NCH_2), 54.3 (PheCH), 127–136 (Phe C_6H_5), 170.3 (PheCONH), 171.5 (CH_3CON), 202.6 (CH_2CO). Mass spectrum, *m/e* M^+ 262. Anal. calcd. for $C_{14}H_{18}N_2O_3$: C, 64.07; H, 6.86; N, 10.67; found: C, 64.01; H, 6.90; N, 10.49.

N-*Acetyl-L-phenylalanyl-(2-oxo-2-phenyl)-ethylamine* ***3d***

Chromatography on silica gel and crystallization from EtOAc gave a white solid: yield 81%; mp 160–162°C; $[\alpha]_D^{20}$ 9° (c=1.0, MeOH) IR: main peaks at 3413, 3320, 2995, 1700, 1660, 1599, 1501, 1450, 1373, 1289 cm^{-1}. ^1H NMR ($CDCl_3$) δ 1.98 (s, 3, CH_3CON), 3.15 (m, 2, $PheCH_2$), 4.71 (d, 2, *J*=4.5 Hz, NCH_2), 4.98 (m, 1, PheCH), 6.72 (d, 1, *J*=8 Hz, AcNH), 7.30 (s, 5, $PheC_6H_5$), 7.30 (t, 1, *J*=4.5 Hz, Phe CONH), 7.55–8 (m, 5, C_6H_5); ^{13}C NMR ($CDCl_3$) δ 23.1 (CH_3CON), 38.6 ($PheCH_2$), 46.3 (NCH_2), 54.5 (PheCH), 127–136 (C_6H_5), 127–136 (Phe C_6H_5), 170.1 (PheCONH), 171.3 (CH_3CON), 193.5 (CH_2CO). Mass spectrum, *m/e* M^+ 324. Anal. calcd. for $C_{19}H_{20}N_2O_3$: C, 70.37; H, 6.17; N, 8.64; found: C, 69.98; H, 6.41; N, 8.52.

N-Acetyl-L-phenylalanylaminoacetaldehyde ***3e***

A mixture of *N*-acetyl-L-phenylalanylallylamide (246 mg, 1 mmol) in EtOAc (3 ml) and osmium tetroxide (13 mg, 0.05 mmol) in water (3 ml) was stirred at room temperature while finely powdered sodium metaperiodate (465 mg, 2.17 mmol) was added in portions over a 40 min period. The tan coloured slurry was stirred for additional 90 min. The mixture was extracted thoroughly with EtOAc, the organic layers washed with saturated brine, combined, dried over Na_2SO_4 and evaporated under reduced pressure to give a pale yellow solid residue (180 mg). After chromatography on silica gel eluting with EtOAc/iPrOH (8:2) the pure aldehyde (148 mg, 58%) was obtained as white solid by trituration with *n*-hexane: mp 134–136°C; $[\alpha]_D^{20}$ 11 (c=1.0, MeOH); IR: main peaks at 3425, 3312, 2998, 1731, 1660, 1604, 1505, 1453, 1377 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.90 (s, 3, CH_3CON), 3.07 (m, 2, $PheCH_2$), 3.97 (d, 2, J= 5.5 Hz, NCH_2), 4.83 (m, 1, PheCH), 7.15 (d, 1, J=8 Hz, AcNH), 7.27 (s, 5, $PheC_6H_5$), 7.65 (t, 1, J=5.5 Hz, PheCONH), 9.53 (s, 1, CHO); ^{13}C NMR ($CDCl_3$) δ 22.4 (CH_3CON), 37.7 ($PheCH_2$), 49.1 (NCH_2), 53.8 (PheCH), 126–138 ($PheC_6H_5$), 169.1 (PheCONH), 172.2 (CH_3CON), 199.7–95.6 ($HC(OH)_2$). Mass spectrum, *m/e* M^+ 248. Anal. calcd. for $C_{13}H_{16}N_2O_3$. 1/2 H_2O: C, 60.68; H, 6.66; N, 10.88; found: C, 60.45; H, 7.03; N, 10.86.

Enzyme purification and assay

Papain (type IV) was purchased from Sigma Chemical Co. The enzyme was further purified from irreversibly inactivated papain as described by Liang and Abeles [34].

Enzyme concentration was determined by titration with 4,4'-dipyridyl disulfide [35] and from the absorbance at 280 nm (E=58.5 mM^{-1} cm^{-1}) [36]. Before each experiment, papain was activated in 50 mM phosphate buffer pH 6.8 containing 2 mM EDTA and 5 mM L-cysteine. The low molecular weight thiol was removed by gel filtration on Sephadex G-25 column (2 × 25 cm) eluted with 50 mM phosphate buffer pH 6.8, 2 mM EDTA. All buffers and solutions were made with ultra high quality water (Elga UHQ) deareated and saturated with O_2-free argon. Solutions of activated papain were stored at 4°C under a continuous stream of O_2-free argon. Under these conditions papain remained in the reduced form for at least 2 h as deduced from titration with 4,4'-dipirydyl disulfide. All molar concentrations of enzyme indicated in the text refer to active enzyme determination by titration with 4,4'-dipyridyl disulfide. All reagents used were from Sigma. Reaction progress curves of papain-catalyzed hydrolysis of ZGlyONp were measured spectrophotometrically by monitoring the release of *p*-nitrophenol at 405 nm [37] in a Kontron double-beam spectrophotometer (Uvikon 860) equipped with a Peltier thermocontroller set at 25°C. A cuvette containing buffer and substrate was used in the reference cell to correct for nonenzymatic hydrolysis of the substrate [37]. The final concentration of organic solvent was 12% (v/v) acetonitrile for compounds **3a–3d** and 10% (v/v) acetonitrile, 1% (v/v) methanol for compounds **4a–4d**. The concentrations of organic solvent are uneffective towards kinetic parameters of papain catalyzed hydrolysis of ZGlyONp [35]. Injection of enzyme solution initiated the reaction. Absorbances were continuously measured and stored in the computerized spectrophotometer. Software was supplied by Kontron. Progress curves were composed of 900–1800 (absorbance, time) pairs. A substrate concentration of 125 μM (K_m×12.5) avoided substrate depletion during all the experiments due to spontaneous and enzymatic hydrolysis. All kinetic parameters indicated in the text were determined by linear regression analysis, but all data were plotted for inspection. Data are the mean of duplicate determinations with SD less than 20%.

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