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Preliminary communication

Synthesis and activity of pyrrolidinyl- and thiazolidinyl-dipeptide derivatives as inhibitors of the Tc80 prolyl oligopeptidase from *Trypanosoma cruzi*

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Abstract – Pyrrolidinyl- and thiazolidinyl- dipeptide derivatives, featuring either a vinyl sulfone-, a 2-ketobenzothiazole-, a nitrile-, or a benzimidazole group at the C-terminus, were designed and synthesized as potential inhibitors of the prolyl-specific Tc80 proteinase from *Trypanosoma cruzi*, the agent of Chagas' disease. These compounds were evaluated in vitro towards the target enzyme which was classified as a serine protease belonging to the prolyl oligopeptidase family (EC 3.4.21.26). A peptidyl nitrile and two peptidyl α-ketobenzothiazoles were shown to be potent reversible and competitive inhibitors of Tc 80 proteinase, with K_i values in the range 38–219 nM, and compared advantageously with some known mammalian prolyl oligopeptidase inhibitors. © 2000 Éditions scientifiques et médicales Elsevier SAS

prolyl oligopeptidase / *Trypanosoma cruzi* / Chagas' disease / inhibitor / peptidyl nitrile / peptidyl α-ketobenzothiazole

1. Introduction

The haemoflagellate *Trypanosoma cruzi* is the causative agent of Chagas' disease in Latin America. Despite vector-control programmes in many of the affected countries, this illness represents a major public health concern notably because of its clinical variability, lack of efficient non-toxic drug and its transmission by infected donor blood.

Entry of *T. cruzi* into non-phagocytic mammalian cells occurs by recruitment and fusion of host lysosomes at the parasite attachment site, an unusual process that results in the formation of a parasitophorous vacuole with lysosomal properties [1]. *T. cruzi* trypomastigotes may also enter macrophages by interfering with the host immune response. Whatever the type of cell being infected, a limited set of *T. cruzi* proteins and target cell components probably play an important role in the parasite host cell relationship. Several proteolytic activities have recently been identified in *T. cruzi* beside the major cysteine proteinase, cruzipain. A 120 kDa serine endopeptidase [2] has been shown to be required for the generation of Ca^{2+} signalling in mammalian cells [3] and an acidic 30 kDa cysteine proteinase was characterized, which exhibited an ability to hydrolyse human type I collagen and bovine serum albumin [4].

A third specific proteinase activity was identified and characterized in cell-free extracts of amastigote, trypomastigote and epimastigote forms of *T. cruzi* using the collagenase-like peptidase fluorogenic substrate *N*-Suc-Gly-Pro-Leu-Gly-Pro-7-amido-4-methylcoumarin (-AMC) [5]. This *T. cruzi* 80 kDa endopeptidase (Tc80 proteinase) is secreted by the infective trypomastigotes. It mediates

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Abbreviations: AMC, 7-amino-4-methylcoumarin; DCC, dicyclohexylcarbodiimide; 3,4-DCI, 3,4-dichloroisocoumarin; DCU, dicyclohexylurea; DFP, diisopropyl fluorophosphate; DIPEA, diisopropylethylamine; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; HOBT, 1-hydroxybenzotriazole; pCMB, p-chloromercuribenzoate; PMSF, phenylmethanesulfonyl fluoride; PyBOP, (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate; pyr, pyrrolidinyl; N-Suc-Gly-Pro-Leu-Gly-Pro-AMC, N-succinyl-glycyl-prolyl-leucyl-glycyl-prolyl-7-amido-4-methylcoumarin; TFAA, trifluoroacetic anhydride; THF, tetrahydrofuran; thia, thiazolidinyl; Z-Leu-Gly-OH, N-benzyloxycarbonyl-leucyl-glycine.

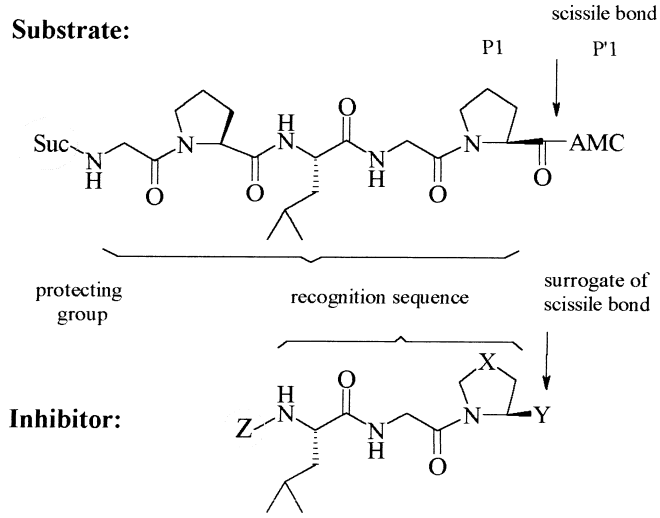
specific degradation of purified human type I and IV collagens, and native type I collagen hydrolysis in rat mesentery at physiological pH. Taken together, the data on this enzyme provide evidence that *T. cruzi* contains a specific proteinase capable of degrading collagens of the extracellular matrix of the cell being infected and suggest that Tc80 proteinase inhibition would represent a therapeutic approach to the treatment of Chagas' disease. At the same time, the development of specific inhibitors would serve as key tools to further investigate the role of the proteinase in the host cell invasion process by *T. cruzi*.

We reasoned that, in the absence of currently known structural requirements concerning the Tc80 proteinase active site, except substrate specificity, a preliminary work aimed at inhibiting the proteinase could be achieved with peptidyl derivatives based on the substrate recognition sequence, the C-terminus of which would have been modified with functional groups likely to interact with the protease active site. In this paper, we report the synthesis and inhibitory properties (IC_{50} and K_i) of a series of derivatives **1**–**5** against Tc80 proteinase, featuring either a vinyl sulfone (**1**), a 2-ketobenzothiazole (**2** and **3**), a nitrile (**4**) or a benzimidazole group (**5**) as surrogate of the P1–P'1 scissile bond in the substrate (*figure 1*). As part of this work, the Tc80 proteinase was classified as a serine protease belonging to the prolyl oligopeptidase family (EC 3.4.21.26) using a standard serine inhibitor, and a limited series of mammalian prolyl oligopeptidase inhibitors was studied and compared to derivatives **1**–**5**.

2. Chemistry

Making use of the well-known properties of Weinreb amides [6, 7], both the peptidyl vinyl sulfone **1** and peptidyl α-ketobenzothiazole **2** could be synthesized from the *N*-methyl-*O*-methyl hydroxamate derivative **6** [8] (*figure 2*). Vinyl sulfone pyrrolidine **9** was obtained through the Wittig-Horner reaction [9] with excellent yield from diethyl phenylsulphonyl methylphosphonate and prolinal **8**, readily avalaible by reduction of **6** with $LiAlH_4$ [10]. Coupling of the *N*-deprotected pyrrolidine **9** with Z-Leu-Gly-OH in the presence of PyBOP/DIPEA reagents provided the desired peptidyl vinyl sulfone **1**.

Compounds **2** and **3** were prepared via nucleophilic addition of the benzothiazole anion to Weinreb amides **6** and **7** [11]. (S)-*N*-methoxy-*N*-methylcarbamoyl-thiazolidine **7** was obtained from the corresponding free carboxylic acid and *N,O*-dimethylhydroxylamine using DCC in 78% yield. Amides **6** or **7** were added to the benzothiazole anion (2 equivalents) in THF at –78 °C. Quenching the reaction mixture with the required amount



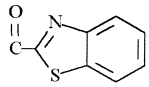
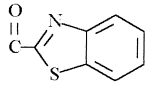
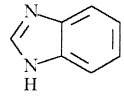
Compound	X	Y
1	CH_2	$CH{=}CHSO_2C_6H_5$
2	CH_2	
3	S	
4	CH_2	CN
5	CH_2	

Figure 1. Tc80 proteinase inhibitors, based on the Leu-Gly-Pro recognition sequence, the C-terminus of which has been replaced by a functional group likely to interact reversibly or irreversibly with the proteinase active site.

of saturated aqueous NH_4Cl for neutralization and subsequent work-up provided α-amino ketones **10** and **11** in 74 and 71% yield, respectively, after silica gel chromatography. Amino alcohols **12** and **13** were obtained through reduction of **10** and **11** by $NaBH_4$ in methanol at –20 °C [12] in 83 and 73% yield with 92 and 95% diastereoselectivity, respectively, and used as a diastereomeric mixture in the following step. After removal of the *t*-butyl group with 5.6 N HCl in AcOEt, the resulting *N*-deprotected amino alcohols were coupled with Z-Leu-

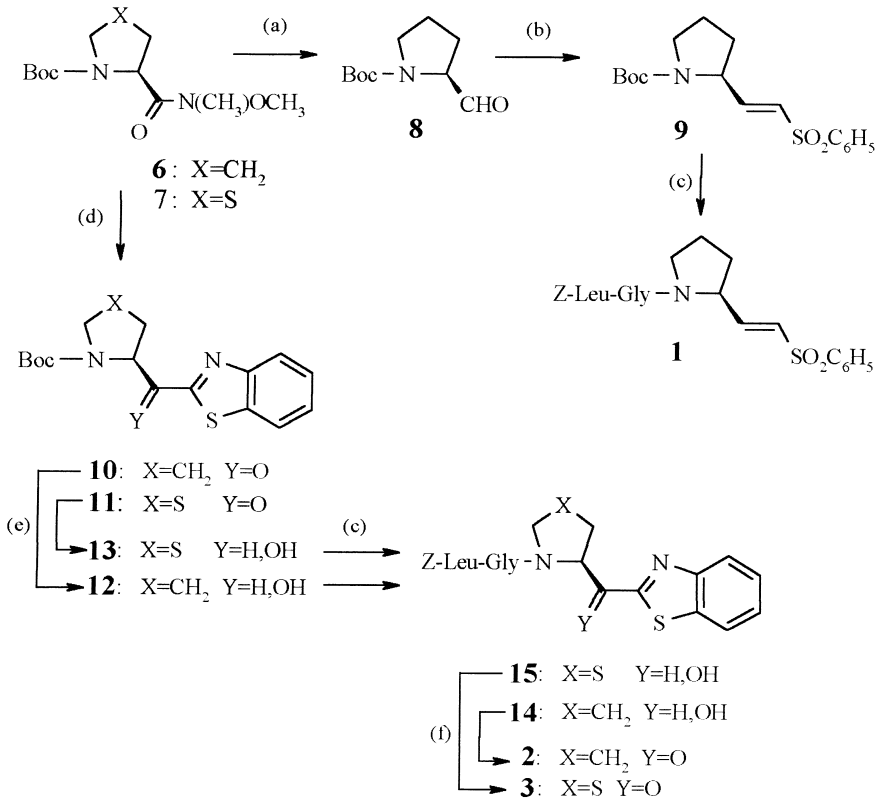


Figure 2. Synthetic scheme for the preparation of peptidyl vinyl sulfone **1** and peptidyl α-ketobenzothiazoles **2** and **3**: (a) $LiAlH_4$, ether; (b) NaH, diethyl phenylsulphonyl methylphosphonate, THF; (c) Z-Leu-Gly-OH, DIPEA, PyBOP, CH_3CN; (d) benzothiazole, BuLi, THF, –78 °C; (e) $NaBH_4$, MeOH, –20 °C; (f) $(COCl)_2$, DMSO, CH_2Cl_2, –60 °C.

Gly-OH in the presence of PyBOP/DIPEA reagents giving the peptidyl alcohols **14** and **15** in 66 and 32% yields, respectively. Finally, Swern oxidation of the hydroxy function provided the expected peptidyl α-ketobenzothiazoles **2** and **3** in 70 and 56% yields, respectively. The ^{13}C-NMR spectrum of compound **2** showed only chemical shifts relevant to the presence of rotamers, indicating that essentially no epimerization occured at the chiral carbon of the five-membered ring during the Weinreb amide alkylation step.

The tripeptidylamide **16** was prepared as a precursor of peptidyl nitrile **4** (*figure 3*), since there are several dehydrating methods to convert amide into nitrile under relatively mild conditions. However, attempts to dehydrate amide **16** according to the Swern oxidation protocol [13] or with Ac_2O- [14] or TFAA-pyridine methods [15, 16] mostly led to recovered starting material. Fortunately, the peptidyl nitrile **4** could be easily obtained by treatment of the same peptidyl amide with $SOCl_2$/DMF in the presence of *N*-methylmorpholine [17] (*figure 3*).

Synthesis of the peptidyl benzimidazole **5** required preparation of pyrrolidinyl benzimidazole **17** which was readily available by condensation of Z-Pro-OH and 1,2-phenylenediamine with DCC/HOBT reagents in THF/CH_2Cl_2 and subsequent cyclization in AcOH at 65 °C [18] (*figure 3*). Hydrogenolysis of compound **17** under H_2 (1 atm.) with 10% Pd/C in MeOH followed by coupling of the resulting *N*-deprotected pyrrolidine and Z-Leu-Gly-OH using DCC/HOBT reagents in THF/CH_2Cl_2 provided the desired peptidyl heterocycle **5** in 82% yield after silica gel chromatography.

3. Biological evaluation

Tc80 proteinase was purified from *T. cruzi* epimastigote forms as previously described [5]. Proteolytic activity was determined using the fluorogenic substrate *N*-Suc-Gly-Pro-Leu-Gly-Pro-AMC. The specific activity of the purified Tc80 proteinase was 0.26 nmol of AMC released/

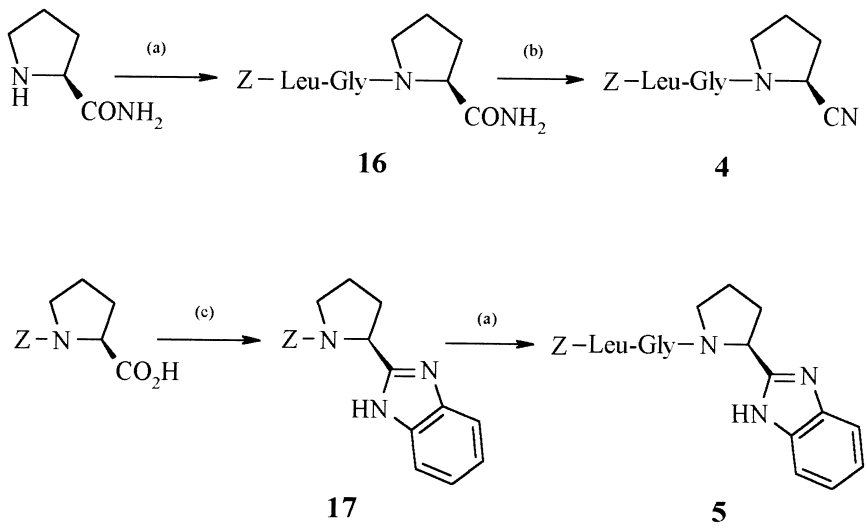


Figure 3. Synthetic scheme for the preparation of peptidyl nitrile **4** and peptidyl benzimidazole **5**: (a) Z-Leu-Gly-OH, HOBT, DCC, THF; (b) N-methylmorpholine, $SOCl_2$, DMF; (c) *(i)* HOBT, 1,2-diaminobenzene, DCC, CH_2Cl_2; *(ii)* AcOH, 65 °C.

min per μg of protein. The IC_{50} value was defined as the inhibitor concentration which causes a 50% decrease of the activity. Data presented are the mean ± standard deviation of at least 3 independent experiments.

Reversibility of the inhibition was evaluated after complete enzyme inhibition by dialysis at 4 °C for 24 h and determination of the recovered enzyme activity. The inhibition constants were determined by the progress curve method in which the inhibitor is added to a steady-state enzyme–substrate reaction [19, 20]. The inhibitor was at least in a 20-fold molar excess over the enzyme. The reactions were carried out under experimental conditions where less than 5% of substrate was hydrolysed. Under these conditions, $v_0/v_i = 1 + [I]/K_{i\ (app)}$ where v_0 is the enzyme activity without inhibitor and v_i the enzyme activity in the presence of inhibitor. $K_{i\ (app)}$ was obtained for several concentrations of inhibitor. The K_i values were calculated from $K_{i\ (app)}$ by the relationship $K_{i\ (app)} = K_i\ (1 + [S]/K_m)$. The K_m value of *N*-Suc-Gly-Pro-Leu-Gly-Pro-AMC (18.5 μM) and the type of inhibition were determined graphically according to the plot [S]/V versus [S] [21].

4. Results and discussion

By its property to cleave peptide bonds at the carboxyl side of proline residues, Tc80 proteinase could be classified as a prolyl oligopeptidase, previously called prolyl endopeptidase or postproline cleaving enzyme. Prolyl oligopeptidase is representative of a new serine peptidase family (EC 3.4.21.26). The enzymes of this family have been reported to have an unusual inhibitor profile characterized by their inactivation by DFP [22, 23] but not by several reagents for the recognition of a serine peptidase (3,4-DCI, PMSF) [24]. Surprisingly, they are also very susceptible to pCMB, a cysteine-protease inhibitor and partially inhibited by the smaller thiol reagent, iodoacetamide. These data suggest the presence of a cysteine residue at or near the active site so that a large reagent attached to this thiol excludes the substrate from the catalytic centre [25–28]. A previous study did not provide conclusive evidence with regard to the Tc80 proteinase class, whether serine- or cysteine-protease. Thus, the enzyme was inhibited by some cysteine reagents (Z-Phe-Ala-CHN_2, pCMB) but remained insensitive to the serine inactivator, PMSF [5]. To clarify the situation with respect to Tc80 proteinase, we examined the effect of DFP on its activity. Indeed, a strong inactivation was observed in the presence of this reagent (IC_{50} value of about 60 nM). On the basis of its overall inhibition profile, the protozoan proteinase can now be unambiguously classified as a serine enzyme belonging to the prolyl oligopeptidase family.

Although the specific activity of the Tc80 proteinase on the pentapeptidyl substrate *N*-Suc-Gly-Pro-Leu-Gly-Pro-AMC was seven-fold higher than that on the dipeptide *N*-Suc-Gly-Pro-AMC [5], we thought that the Leu-Gly-Pro sequence would provide a sufficient potential binding

for the purpose of our study. Inhibitors were designed by replacing the C terminus of the P3–P1 sequence by a functional group, Y, likely to interact irreversibly (**1**, Y: vinyl sulfone) or reversibly (**2**, **3**, Y: 2-ketobenzothiazole; **4**, Y: nitrile; **5**, Y: benzimidazole) at the active site of the enzyme (*figure 1*). Vinyl sulfone as a Michael acceptor specific for cysteine proteinase was first described by Liu and Hanzlik [29] and since developed towards several thiol peptidases. The presumed presence of a cysteine residue near the active site of the Tc80 proteinase and the reported covalent modifications of the reactive cysteine thiol- and serine hydroxy groups of the mammalian prolyl oligopeptidase by an O-acylhydroxamate derivative and Ac-Ala_2-Pro-CHN_2, respectively, both thiol protease specific inhibitors, [30, 31], prompted us to examine the effect of a peptidyl vinyl sulfone on the Tc80 peptidase activity. We retained nitrile and 2-ketobenzothiazole groups for similar reasons. The peptidyl α-keto-heterocycle concept was introduced by Edwards and co-workers towards the serine protease elastase [32], and subsequently applied to cysteine- and other serine-proteases including prolyl endopeptidase [33]. Nitrile derivatives have also been reported to exert potent inhibitory effects towards thiol proteases and prolyl oligopeptidase, possibly by mimicking the initial covalent enzyme adduct through a thioimidate and imidate function, respectively [34]. Lastly, we planned to investigate the effect of a benzimidazole moiety which may be regarded as a hydrolytically stable peptide bond isostere with both a potential hydrogen acceptor and a donor [35].

The functionalized peptides **1**–**5** were easily prepared by liquid phase synthesis. Compounds **1**–**3** were obtained using the Weinreb amide methodology, with alkylation of the appropriate *N*-methyl-*N*-methoxy amide by lithiated benzothiazole as a key step in the case of **2** and **3** [11]. The nitrile group in **4** and the benzimidazole moiety in **5** were introduced by standard methods.

The IC_{50} values for Tc80 inhibition by the compounds synthesized are listed in *table I*. Peptidyl α-ketobenzothiazoles **2**, **3** and nitrile **4** were revealed as reversible inhibitors since after a complete enzyme inhibition by 10 μM of each derivative, an extensive dialysis by ultrafiltration allowed the recovery of more than half of the initial enzymatic activity. These three derivatives displayed competitive inhibition characteristics as determined by the plots [S]/V versus [S] [21]. Kinetics of inhibition of Tc80 proteinase by compound **4** are depicted in *figure 4*. The intercept on the [S]/V axis (Km/V) increased with increasing concentrations of inhibitor, and equivalent slopes (1/V) were determined, indicating a competitive inhibition. Similar data were obtained with the 2-ketobenzothiazole derivatives **2** and **3** (data not shown). K_i values of compounds **2**, **3** and **4** were 219 ± 28, 139 ± 64 and 38 ± 14 nM, respectively (*table I*). According to our data, the binding mode was fast since no difference in potency was found with or without preincubation of the enzyme with the inhibitors. Nitrile derivative **4** was the most potent inhibitor of the Tc80 proteinase activity.

Table I. Inhibition of Tc80 proteinase activity by compounds **1–5**, **14** and three mammalian prolyl oligopeptidase inhibitors: IC_{50} and K_i values.

Compound	IC_{50} (nM)	K_i (nM)
1	> 10 000	35 000
2	200 ± 27	219 ± 28
3	251 ± 22	139 ± 64
4	52 ± 17	38 ± 14
5	> 10 000	n.d.[a]
14	> 10 000	n.d.
Z-Pro-L-prolinal dimethyl acetal	6 700	1 120 ± 120
Boc-Asn-Phe-Pro-aldehyde	4 300	280 ± 110
Z-Pro-Pro-OH	> 10 000	n.d.

Data are the mean ± standard deviation of at least 3 independent experiments except for the IC_{50} values of Z-Pro-L-prolinal dimethyl acetal and Boc-Asn-Phe-Pro-aldehyde which are the mean of 2 independent experiments. [a]n.d.: not determined.

The introduction of a sulfur atom in the ring of several pyrrolidinyl derivative inhibitors of mammalian prolyl

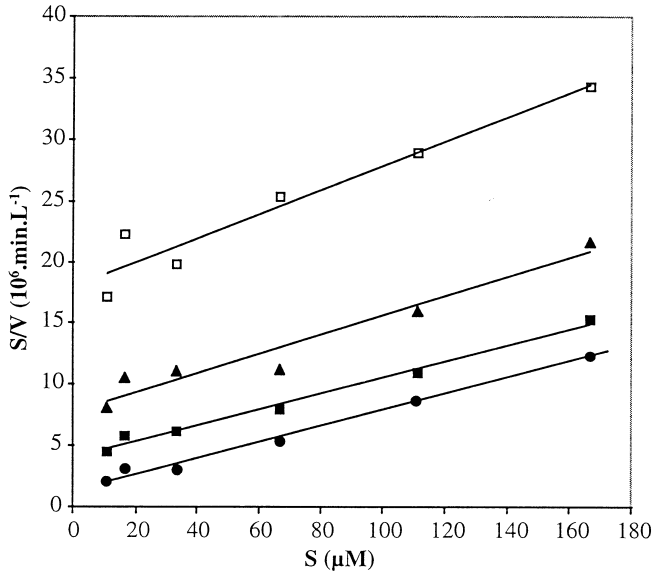


Figure 4. Cornish-Bowden plots [21]: determination of the inhibition type of the Tc80 proteinase by compound **4** at 333.3 nM (□), 111 nM (▲), 33.3 nM (■), and in the absence of **4** (●).

oligopeptidase has been reported to afford compounds with remarkably increased inhibition potency but, unfortunately, we did not notice a significant increase when evaluating thiazolidinyl compound **3** compared to **2**. Compound **1** exhibited only a weak inhibition of the Tc80 peptidase, even after a lengthy preincubation (K_i = 35 000 nM). The vinyl sulfone group could be mispositioned for reacting with the putative cysteine near the active centre. While our work was in progress, an X-ray study of the porcine prolyl oligopeptidase complexed with a peptidyl aldehyde was reported in which this cysteine residue was identified as Cys 255 and situated closer to the S3- than the S1-subsite [36]. Not surprisingly, hydroxy compound **14** did not inhibit Tc80 proteinase activity (IC_{50} values > 10 μM) [37]. The absence of inhibition activity observed wih **5** (IC_{50} value > 10 μM) suggests that the imidazole moiety poorly mimics the scissile bond: the oxyanion hole of the enzyme might not accommodate the imine nitrogen instead of C=O due to electronic effects and steric hindrance. Inhibitory activity of **2** and **3** compared to **5** strongly suggests that the presence of a carbonyl α to the heterocycle is essential for an interaction to take place in the active site. Several mammalian prolyl oligopeptidase inhibitors are now available, three of them, Z-Pro-L-prolinal dimethyl acetal [38], Boc-Asn-Phe-Pro-H (unpublished data), and Z-Pro-Pro-OH [37] were also tested against Tc80 peptidase (*table I*). Boc-Asn-Phe-Pro-H (K_i = 280 ± 110 nM) is the most potent inhibitor of this limited series, however its K_i value remains 5–6-fold higher than that of **4** and slightly higher than that of **2** and **3**.

The three newly synthesized compounds, **2**–**4**, likely to act by transition state analogue adduct formation, exhibit potent reversible and competitive inhibition of the Tc80 proteinase. Collectively, the findings presented herein are consistent with classifying this *T. cruzi* peptidase as a member of the prolyl oligopeptidase family. Our results, together with those disclosed recently by a combinatorial approach towards Tc80 proteinase [39, 40], may serve to design new potent inhibitors of this enzyme. The study of the novel synthetic inhibitors **2**–**4** towards the *T. cruzi*-cell invasion process is under way.

5. Experimental protocols

5.1. Chemistry

Melting points are uncorrected. IR spectra were recorded in the FT-mode. ^{1}H- and ^{13}C-NMR spectra were recorded at 300 and 75 MHz, respectively, and only chemical (δ) shifts of the major rotamers are reported (ppm relative to solvent signal as the internal reference). Analyses indicated by the symbols of the elements were within ± 0.4% of theoretical values. Mass spectra were recorded on a Jeol MS 700 apparatus. TLC analyses were performed on thin layer analytical plates $60F_{264}$ (Merck). Flash column chromatography was performed on silica gel 60 (Merck).

5.1.1. 3-tert-*Butyloxycarbonyl-4(S)-N-methyl-N-methoxycarbamoyl-1,3-thiazolidine* **7**

To a suspension of *N,O*-dimethylhydroxylamine hydrochloride (1.844 g, 18.9 mmol) in dry CH_2Cl_2 (30 mL) at 5 °C under argon, was added successively, neat DIPEA (2.44 g, 18.9 mmol), *N*-Boc-L-thiazolidine-4-carboxylic acid (2.517 g, 18.9 mmol) and DCC (4.094 g, 19.8 mmol) as solids. The mixture was stirred overnight at room temperature, DCU was filtered off, the filtrate was concentrated and the residue was taken up in ether (40 mL). The organic phase was washed successively by 1 N HCl (2 × 10 mL), H_2O (10 mL), saturated $NaHCO_3$ and brine before flash chromatography on silica gel (pentane; ether/pentane, 1:3; 1:2, 4:5, 1:1): colourless oil 2.159 g (78.2%), $[\alpha]^{20}_D$ –103 (c = 4.7 AcOEt); ^{1}H-NMR ($CDCl_3$) δ 1.43 (s, 9H, *tert*-C_4H_9); 2.97–3.08 (m, 1H, 5-CH(thia)); 3.18 (s, 3H, NCH_3); 3.30–3.43 (m, 1H, 5-CH(thia)); 3.73 (bs, 1H, OCH_3); 4.47–4.50 (m, 1H, 2-C<u>H</u>H(thia)); 4.63–4.76 (m, 1H, 2-C<u>H</u>H(thia)), 5.07 (m, 1H, 4-CH(thia)); ^{13}C-NMR ($CDCl_3$) δ 26.51, 32.13, 33.09, 49.11, 59.06, 61.06, 80.47, 152.90, 170.93. Anal. $C_{11}H_{20}N_2O_4S$ (C, H, N).

5.1.2. 1-tert-*Butyloxycarbonyl-2(S)-[phenylsulphonyl-(E)-ethenyl]pyrrolidine* **9**

To NaH (92 mg, 60% dispersion in oil, 2.3 mmol) suspended in dry THF (3 mL), was slowly added dropwise a solution of diethyl phenylsulphonylmethylphosphonate (598 mg, 2.3 mmol) in THF (3 mL) under argon at room temperature. When hydrogen had evolved, the mixture was stirred for an additional 10 min and a solution of freshly prepared Boc-L-prolinal [8] (458 mg, 2.3 mmol) in THF (3 mL) was added. After stirring for 1 h, ice cold water was added (20 mL), the mixture was extracted with AcOEt (5 × 10 mL) and the combined organic layers were dried ($MgSO_4$). The crude product was flash chromatographed on silica gel (ether/pentane, 1:1): 590 mg (76%) white solid, m.p. 82–83 °C; $[\alpha]^{20}_D$ –39.4 (c = 2 AcOEt); IR (KBr): 1 683, 1 478, 1 404, 1 367, 1 309 cm^{-1}; ^{1}H-NMR δ 1.15 (s, 9H, *tert*-C_4H_9), 1.74–1.90 (m, 3H, 3-CH_2(pyr), 4-CH(pyr)), 2.00–2.18 (m, 1H, 3-CH(pyr)), 3.28–3.46 (m, 2H, 5-CH_2(pyr)), 4.28–4.41 (m, 1H, 2-CH(pyr)), 6.27 (d, *J* = 14.0 Hz, C=C<u>H</u>SO_2), 6.80 (dd, *J* = 5.4, 15.0 Hz, C<u>H</u>=CSO_2), 7.44–7.65 (m, 3H_{arom}), 7.86 (d, *J* = 7.2 Hz; 2H_{arom}); MS

m/z = 281 ($MH^{+\cdot}$–C_4H_9), 205 ($M^{+\cdot}$–C_4H_9O), 205, 170, 149, 114, 95, 71, 58, 42. Anal. $C_{17}H_{23}NO_4S$ (C, H, N).

*5.1.3. 2-[Benzo[d][1,3]thiazol-2-yl-carbonyl]-1-*tert*-butyloxycarbonyl-(2S)pyrrolidine* **10**

To benzothiazole (812 mg, 6 mmol) in dry THF (7 mL) at –78 °C under argon, was added dropwise BuLi (1.6 M, 6 mmol). The mixture was stirred for 10 min after the end of addition and a solution of *N*-methoxy-*N*-methyl-N_α-Boc-L-prolinamide [8] (520 mg, 2 mmol) in THF (2 mL) was added dropwise, maintaining the temperature at –78 °C. After stirring for an additional 30 min at the same temperature, the reaction mixture was quenched with saturated aqueous NH_4Cl (20 mL) and allowed to warm to 0 °C before partitioning with AcOEt (20 mL). The organic layer was quickly washed with chilled 1 N HCl (3 × 6 mL), water (3 mL), saturated $NaHCO_3$ (3 × 3 mL) and dried ($MgSO_4$). Purification by flash chromatography on silica gel (cyclohexane/AcOEt, 98:2; 96:4) yielded the expected compound, 557 mg (84%), as a yellowish solid, m.p. 81–84 °C; $[\alpha]^{20}_D$ –24.5 (c = 2.1 MeOH); IR (KBr) 1 705, 1 558, 1 485 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 1.17 (s, 9H, *tert*-C_4H_9) 1.89 (m, 2H, 4-CH_2(pyr)), 1.99–2.40 (m, 2H, 3-CH_2(pyr)), 3.53 (m, 2H, 5-CH_2(pyr)), 5.54 (dd, *J* = 4.9, 7.9 Hz, 1H, 2-CH(pyr)), 7.48 (m, $2H_{arom}$), 7.90 (m, $1H_{arom}$), 8.10 (m, $1H_{arom}$); MS m/z = 332 ($M^{+\cdot}$), 259, 232, 170, 166, 152, 135, 114. HRMS calcd. for $C_{17}H_{20}N_2O_3S$, 332.11946; found, 332.11945.

*5.1.4. 4-[Benzo[d][1,3]thiazol-2-yl-carbonyl]-3-*tert*-butyloxycarbonyl-1,3-(4S)-thiazolidine* **11**

This compound was obtained as described for the preparation of **10** except starting from *N*-methyl-*N*-methoxy amide **7** (1.38 g, 5 mmol) instead of *N*-methoxy-*N*-methyl-N_α-Boc-L-prolinamide. Flash chromatography on silica gel (cyclohexane; AcOEt/cyclohexane 0.5:99.5; 1:99; 1.25:98.75; 1.5:98.5; 2:98): yellowish oil, 1.755 g, (71.6%); $[\alpha]^{20}_D$ –35.5 (c = 5.8 AcOEt); IR (KBr) 3 407, 1 702, 1 663 cm^{-1}; ^{1}H-NMR ($CDCl_3$) δ 1.26 (s, 9H, *tert*-C_4H_9), 3.33 (dd, *J* = 4.8, 12.1 Hz, 1H, 5-CH<u>H</u>(thia)), 3.61–3.69 (m, 1H, 5-CHH(thia)), 4.59–4.78 (m, 2H, 2-CH_2(thia)), 5.83 (m, 1H, 4-CH(thia)), 7.5–7.62 (m, $2H_{arom}$), 7.96 (m, $1H_{arom}$), 8.14 (m, $1H_{arom}$); ^{13}C-NMR ($CDCl_3$) δ 27.72, 34.71, 49.61, 64.08, 80.73, 122.11, 125.21, 126.91, 127.78, 136.73, 152.60, 153.05, 163.48, 190.40; MS (DCI) m/z = 351 ($MH^{+\cdot}$). DCI-HRMS calcd. for $C_{16}H_{19}N_2O_3S_2$, 351.0837; found, 351.0837.

*5.1.5. 2-[Benzo[d][1,3]thiazol-2-yl-hydroxymethyl]-1-*tert*-butyloxycarbonyl-(2S)-pyrrolidine* **12**

A solution of compound **10** (166 mg, 0.5 mmol) in dry MeOH (4 mL) cooled to –20 °C under argon was treated with $NaBH_4$ (20 mg, 0.55 mmol). Stirring was maintained for 40 min during which the temperature was allowed to warm to –10 °C and the mixture was quenched with water and quickly extracted with ether (8 × 4 mL). After washing with brine (2 × 4 mL) and drying ($MgSO_4$), the title compound was obtained as an amorphous solid, 140 mg (83%), m.p. 132–133 °C, and used for the next reaction without further purification. ^{1}H-NMR ($CDCl_3$) δ 1.50 (s, 9H, *tert*-C_4H_9), 1.54–2.24 (m, 4H, 3-CH_2(pyr) and 4-CH_2(pyr)), 3.40–3.53 (m, 2H, 5-CH_2(pyr)), 4.19 (m, 1H, 2-CH(pyr)), 4.97 (m, 1H, C<u>H</u>OH), 6.60 (bs, 1H, CHO<u>H</u>), 7.36–7.45 (m, $2H_{arom}$), 7.87–7.98 (m, $2H_{arom}$).

*5.1.6. 4-[Benzo[d][1,3]thiazol-2-yl-hydroxymethyl]-3-*tert*-butyloxycarbonyl-1,3-(4S)-thiazolidine* **13**

Compound **11** (350 mg, 1 mmol) was submitted to the conditions described in the preparation of **12**. Flash chromatography (cyclohexane; AcOEt/cyclohexane, 10:90; 1:6): 256 mg (73%) as a yellowish oil; $[\alpha]^{20}_D$ –25.6 (c = 5.6 AcOEt); ^{1}H-NMR ($CDCl_3$) δ 1.46 (s, 9H, *tert*-C_4H_9), 3.04–3.15 (m, 1H, 5-CH<u>H</u>(thia)), 3.32–3.46 (m, 1H, 5-CHH(thia)), 4.31 (d, *J* = 9.2 Hz, 1H, C<u>H</u>OH), 4.58–4.69 (m, 2H, 2-CH_2(thia)), 5.20 (m, 1H, 4-CH(thia)), 6.32 (bs, 1H, CHO<u>H</u>), 7.33–7.47 (m, $2H_{arom}$), 7.88 (m, $1H_{arom}$), 7.96 (m, $1H_{arom}$); ^{13}C-NMR ($CDCl_3$) δ 26.42, 27.68, 49.03, 64.01, 72.92, 80.71, 121.27, 122.42, 124.54, 125.49, 134.45, 152.40, 152.59, 173.91; MS (DCI) m/z = 353 ($MH^{+\cdot}$). DCI-HRMS calcd. for $C_{16}H_{21}N_2O_3S_2$, 353.0994; found, 353.0996.

5.1.7. 2-[Benzo[d][1,3]thiazol-2-yl-hydroxymethyl]-1-(N-benzyloxycarbonyl-leucyl-glycyl)-(2S)-pyrrolidine **14**

Compound **12** (150 mg, 0.45 mmol) was treated by HCl/AcOEt (5.6 N, 2 mL) for 10 min at 4 °C. The hydrochloride obtained after evaporation of the solvent under vacuum was taken up in CH_3CN (3.5 mL) to which were added successively, Z-Leu-Gly-OH (153 mg, 0.47 mmol) as a solid, neat DIPEA (241 µL, 1.41 mmol) and PyBOP (234 mg, 0.45 mmol) as a solid at 4 °C under argon. After stirring for 1 h, the mixture was concentrated under reduced pressure, taken up by AcOEt (10 mL), washed with 1 N HCl (3 × 2 mL), water (2 mL), saturated $NaHCO_3$ (2 mL) and dried ($MgSO_4$). Flash chromatography on silica gel (CH_2Cl_2; CH_2Cl_2/MeOH, 99.5:0.5; 99:1; 98.5:1.5): 160 mg (66%) as an amorphous solid, m.p. 79–81 °C; $[\alpha]^{20}_D$ –50.8 (c = 2 AcOEt); ^{1}H-NMR ($CDCl_3$) δ 0.89 (d, *J* = 5.7 Hz, 6H, $C(CH_3)_2$), 1.44–2.18 (m, 7H, β-CH_2(Leu), γ-CH(Leu), 3-CH_2(pyr), 4-CH_2(pyr)), 3.06–3.32 (m, 2H, 5-CH_2(pyr)), 3.95–4.17 (m, 2H, CH_2(Gly)), 4.47 (m, 2H, α-CH(Leu), 2-CH(pyr)), 4.99–5.10 (m, 2H, CH_2Ar), 5.28 (m, 1H, C<u>H</u>OH), 5.76 (bs, 1H, CHO<u>H</u>), 5.97 (d, *J* = 8.4 Hz, 1H,

NH), 6.15 (d, *J* = 3.8 Hz, 1H, NH), 7.21–7.40 (m, 7H$_{arom}$), 7.80–7.92 (m, 2H$_{arom}$).

5.1.8. 4-[Benzo[d][1,3]thiazol-2-yl-hydroxymethyl]-3-(N-benzyloxycarbonyl-leucyl-glycyl)-1,3-(4S)-thiazolidine **15**

Compound **13** (146 mg, 0.42 mmol) was submitted to the conditions described in the preparation of **14**. Flash chromatography on silica gel (CH_2Cl_2; CH_2Cl_2/MeOH, 99.6:0.4; 99.4:0.6; 99.2:0.8): 76 mg (32%) as an amorphous solid, m.p. 81–83 °C; $[\alpha]^{20}_D$ –33.7 (c = 1.1 AcOEt); ^{1}H-NMR ($CDCl_3$) δ 0.90 (d, *J* = 5.2 Hz, 6H, C(C$\underline{H}_3$)$_2$), 1.48–1.69 (m, 3H, β-CH$_2$(Leu), γ-CH(Leu)), 2.99–3.49 (m, 2H, 5-C$\underline{H}_2$(thia)), 4.02–4.86 (m, 7H, C$\underline{H}_2$(Gly), α-CH(Leu), 2-CH$_2$(thia) and 4-CH(thia)), 5.01-5.10 (m, 2H, CH$_2$Ar), 5.31 (m, 1H, C$\underline{H}$OH), 5.64 (bs, 1H, CHO$\underline{H}$), 5.68 (bs, 1H, NH), 5.95 (bs, 1H, NH), 7.26–7.48 (m, 7H$_{arom}$), 7.84–7.97 (m, 2H$_{arom}$); MS (DCI) m/z = 557 ($MH^{+\cdot}$). DCI-HRMS calcd. for $C_{27}H_{33}N_4O_5S_2$, 557.1892; found, 557.1893.

5.1.9. N_α-Benzyloxycarbonyl-leucyl-glycyl-prolinamide **16**

To a suspension of L-prolinamide (228 mg, 2 mmol) in dry THF (4 mL) at 4 °C under argon, were successively added, neat Z-Leu-Gly-OH (644 mg, 2 mmol), neat HOBT (540 mg, 4 mmol) and a solution of DCC (434 mg, 2.1 mmol) in THF (2 mL). After being stirred for 1 h at 4 °C, the whole was concentrated under vacuum, the residue was taken up with CH_2Cl_2 (15 mL) and DCU was filtered off. The filtrate was washed successively with 1 N HCl (3 × 4 mL), water (4 mL), saturated $NaHCO_3$ (3 × 4 mL) and brine (4 mL), dried ($MgSO_4$) and flash chromatographed on silica gel (CH_2Cl_2; CH_2Cl_2/MeOH, 99:1; 98:2; 97:3): 614 mg (73%) as a white solid, m.p. 87–89 °C; $[\alpha]^{20}_D$ –68.7 (c = 2, CH_2Cl_2); ^{1}H-NMR ($CDCl_3$) δ 0.87 (d, *J* = 5.9 Hz, 6H, C(CH$_3$)$_2$), 1.49–2.14 (m, 7H, β-CH$_2$(Leu), γ-CH(Leu), β-CH$_2$(Pro), γ-CH$_2$(Pro)), 3.34–3.58 (m, 2H, δ-CH$_2$(Pro)), 4.00 (d, *J* = 3.7 Hz, 2H, CH$_2$(Gly)), 4.31–4.42, (m, 2H, α-CH(Leu), α-CH(Pro)), 4.99–5.10 (m, 2H, CH$_2$Ar), 5.78 (d, *J* = 8.0 Hz, 1H, NH(Leu)), 6.18 (bs, 1H, NH), 6.84 (bs, 1H, NH), 7.28 (s, 5H$_{arom}$), 7.64 (bs, 1H, NH). Anal. $C_{21}H_{30}N_4O_5.0.5H_2O$ (C, H, N).

5.1.10. 2-Benzimidazol-2-yl-1-benzyloxycarbonyl-(2S)-pyrrolidine **17**

To Z-Pro-OH (498 mg, 2 mmol) in CH_2Cl_2 (6 mL) were added successively, a solution of HOBT (604 mg, 4 mmol) in THF (6 mL), neat 1,2-phenylenediamine (216 mg, 2 mmol) and a solution of DCC (452 mg, 2.2 mmol) in CH_2Cl_2 (5 mL). After being stirred for 4h, the whole was filtered and the crude product was purified through a silica pad (CH_2Cl_2/MeOH, 99.5:0.5), providing *N*-(2-aminophenyl)-N_α-Z-prolinamide which was treated by AcOH at 65 °C for 90 min. After evaporation of the solvent under reduced pressure, the residue was taken up by AcOEt and washed with saturated $NaHCO_3$. Flash chromatography on silica gel (CH_2Cl_2; CH_2Cl_2/MeOH, 99.5:0.5) yielded the title compound as a white solid, 397 mg (61%), m.p. 93–94 °C; $[\alpha]^{20}_D$ –109.2 (c = 2 AcOEt); ^{1}H-NMR ($CDCl_3$) δ 1.54–2.29 (m, 4H, 3-CH$_2$(pyr), 4-CH$_2$(pyr)), 3.04 (m, 1H, 2-CH(pyr)), 3.47–3.60 (m, 2H, 5-CH$_2$(pyr)), 5.12–5.25 (m, 2H, CH$_2$Ar), 7.14–7.33 (m, 8H$_{arom}$), 7.70 (bs, 1H, NH). Anal. $C_{19}H_{19}N_3O_2.0.5H_2O$ (C: calcd 69.07 found 69.67, H, N).

5.1.11. 1-(N-Benzyloxycarbonyl-leucyl-glycyl)-2(S)-[phenylsulphonyl-(E)-ethenyl]pyrrolidine **1**

Obtained according to the procedure described for the preparation of **14** except starting from compound **9** (168 mg, 0.5 mmol) instead of **12**. Flash chromatography on silica gel (AcOEt/cyclohexane, 97:3) yielded the title compound **1** as an amorphous solid, 100 mg (40%), m.p. 62–63 °C; $[\alpha]^{20}_D$ –85.1 (c = 1 MeOH); ^{1}H-NMR (DMSO-d_6) δ 0.84 (d, *J* = 5.7 Hz, 6H, C(CH$_3$)$_2$), 1.41–1.47 (m, 1H, γ-CH(Leu)), 1.56–2.01 (m, 6H, β-CH$_2$(Leu), 3-CH$_2$(pyr), 4-CH$_2$(pyr)), 3.31–3.59 (m, 2H, 5-CH$_2$(pyr)), 3.77–3.98 (m, 2H, CH$_2$(Gly)), 4.03–4.25 (m, 1H, α-CH(Leu)), 4.63–4.68 (m, 1H, 2-CH(pyr), 5.00 (s, 2H, CH$_2$Ar), 6.73 (d, *J* = 15 Hz, C=CHSO$_2$), 6.85 (dd, *J* = 4.5, 15 Hz, 1H, CH=CSO$_2$), 6.90 (bs, 1H, NH), 7.42–7.94 (m, 6H, 5H$_{arom}$, NH). FAB-HRMS calcd. for $C_{28}H_{36}O_6N_3S$, 542.2325; found, 542.2310.

5.1.12. 2-[Benzo[d][1,3]thiazol-2-yl-carbonyl]-1-(benzyloxycarbonyl-leucyl-glycyl)-(2S)pyrrolidine **2**

To a solution of oxalyl chloride (10 μL, 0.12 mmol) in CH_2Cl_2 (300 μL) at –60 °C under argon, was added a solution of dry DMSO (18 μL, 0.24 mmol) in CH_2Cl_2 (36 μL). Stirring was maintained for 10 min, then a solution of compound **14** (22 mg, 0.04 mmol) in CH_2Cl_2 (100 μL), was added and the mixture was stirred for a further 30 min before treatment with triethylamine (46 μL, 0.33 mmol). The resulting slurry was quickly warmed to 0 °C, stirred for 4 h at that temperature before being taken up in AcOEt (10 mL), washed with brine and dried. Purification on silica-gel thin layer chromatography (CH_2Cl_2/MeOH, 99:1) yielded the title compound as an amorphous solid, 15 mg (70%), m.p. 73–74 °C; $[\alpha]^{20}_D$ –55.7 (c = 1.5 MeOH); ^{1}H-NMR ($CDCl_3$) δ 0.87 (d *J* = 5.8 Hz, 6H, C(CH$_3$)$_2$), 1.51–1.76 (m, 3H, β-CH$_2$(Leu), γ-CH(Leu)), 2.06–2.51 (m, 4H, 3-CH$_2$(pyr), 4-CH$_2$(pyr)), 3.54–3.71 (m, 2H, 5-CH$_2$(pyr)), 4.00–4.22 (m, 4H, CH$_2$(Gly), α-CH(Leu), NH), 5.00–5.10 (m, 2H, CH$_2$Ar),

5.22 (d J = 8.2 Hz, 1H, NH), 5.82 (d, J = 3.9, 8.6 Hz, 1H, 2-CH(Pyr)), 6.89 (bs, 1H, NH), 7.25–7.30 (m, $5H_{arom}$), 7.49–7.60 (m, $2H_{arom}$), 7.95–8.00 (m, $1H_{arom}$), 8.15–8.20 (m, $1H_{arom}$); ^{13}C-NMR ($CDCl_3$) δ 21.71, 22.90, 24.66, 29.11, 41.90, 41.96, 46.14, 53.54, 61.82, 66.96, 122.40, 125.64, 127.06, 127.91, 128.01, 128.04, 136.21, 137.29, 153.53, 156.01, 164.11, 166.39, 172.20, 191.78. FAB-HRMS calcd. for $C_{28}H_{33}N_4O_5S$, 537.2172; found, 537.2181.

5.1.13. 4-[Benzo[d][1,3]thiazol-2-yl-carbonyl]-3-(benzyloxycarbonyl-leucyl-glycyl)-1,3-(4S)-thiazolidine **3**

Compound **15** (27 mg, 0.048 mmol) was submitted to the conditions described in the preparation of **2**. Purification on silica-gel thin layer chromatography (CH_2Cl_2/MeOH, 99:1): 9 mg (34%) whitish solid, m.p. 73–75 °C; $[\alpha]^{20}_D$ –38.6 (c = 0.9 MeOH); ^{1}H-NMR ($CDCl_3$) δ 0.89 (d, J = 5.2 Hz, 6H, C ($C\underline{H}_3)_2$), 1.45–1.70 (m, 3H, β-CH_2(Leu), γ-CH(Leu)), 3.35–3.43 (m, 1H, 5-C$\underline{H}$H(thia)), 3.61–3.76 (m, 1H, 5-C$\underline{H}$H(thia)), 4.05–4.32 (m, 1H, α-CH(Leu)), 4.64–4.70 (m, CH_2(Gly)), 4.90–5.18 (m, 4H, CH_2Ar, 2-CH_2(thia)), 6.18 (m, 1H, 4-CH(thia)), 6.83 (bs, 1H, NH), 7.30 (m, $5H_{arom}$), 7.51–7.61 (m, $2H_{arom}$), 7.97–8.19 (m, $2H_{arom}$); MS (DCI) m/z = 555 ($MH^{+\cdot}$). DCI-HRMS calcd. for $C_{27}H_{31}N_4O_5S_2$, 555.1736; found, 555.1735.

5.1.14. 1-(N-Benzyloxycarbonyl-leucyl-glycyl)-2-cyanopyrrolidine **4**

To tripeptide amide **16** (126 mg, 0.3 mmol) in dry DMF (0.4 mL) at 4 °C, were added successively, N-methylmorpholine (92 μL, 2.8 eq.) and $SOCl_2$ (44 μL, 2 eq.). The mixture was stirred for 2h at room temperature and poured onto a chilled saturated $NaHCO_3$ solution (10 mL). After extraction with AcOEt (4 × 2 mL), the combined organic phases were washed with 1 N HCl (4 × 2 mL), water (2 mL), brine (2 mL) and dried ($MgSO_4$). Purification by flash chromatography (CH_2Cl_2; CH_2Cl_2/MeOH, 99.5:0.5) yielded the expected peptidyl nitrile **4** as an amorphous solid, 69 mg, (57%), m.p. 57–59 °C; $[\alpha]^{20}_D$ –82.9 (c = 2.6 CH_2Cl_2); ^{1}H-NMR ($CDCl_3$) δ 0.89 (d, J = 5.9 Hz, 6H, $C(CH_3)_2$), 1.48–1.65 (m, 3H, β-CH_2(Leu), δ-CH-(Leu)), 2.01–2.24 (m, 4H, 3-CH_2(pyr), 4-CH_2(pyr)), 3.35–3.56 (m, 2H, 5-CH_2(pyr)), 3.90–4.10 (m, 2H, CH_2(Gly)), 4.33 (m, 1H, α-CH(Leu)), 4.70 (m, 1H, 2-CH(pyr)), 5.00–5.10 (m, 2H, CH_2Ar), 5.68 (d, J = 8.1 Hz, 1H, NH(Leu)), 7.29 (s, 6H, $5H_{arom}$, NH). FAB-HRMS calcd. for $C_{21}H_{29}N_4O_4$ 401.2189; found 401.2227.

5.1.15. 2-Benzimidazol-2-yl-1-(N-benzyloxycarbonyl-leucyl-glycyl)-(2S)- pyrrolidine **5**

Compound **17** (76 mg, 0.24 mmol) was hydrogenolysed for 2 h under H_2 (1 atm.) using 10% Pd/C in MeOH. After filtration and evaporation to dryness, the free amine was treated for coupling with Z-Leu-Gly-OH using the procedure described for the preparation of **9**. Flash chromatography on silica gel (CH_2Cl_2; CH_2Cl_2/MeOH, 99:1; 98:2; 97:3; 96:4) provided the title compound **5** as an amorphous solid, 96 mg (82%); m.p. 103–105 °C; $[\alpha]^{20}_D$ –120.8 (c = 2 AcOEt); ^{1}H-NMR δ 0.91 (d, J = 5.8 Hz, 6H, $C(CH_3)_2$), 1.47–2.40 (m, 7H, β-CH_2(Leu), γ-CH(Leu), 3-CH_2(pyr), 4-CH_2(pyr)), 2.97 (m, 1H, 2-CH(pyr)), 3.42–3.54 (m, 2H, 5-CH_2(pyr)), 3.82–4.04 (m, 2H, CH_2(Gly)), 4.30 (m, 1H, α-CH(Leu)), 5.10 (s, 2H, CH_2Ar), 5.27 (d, J = 6.8 Hz, 1H, NH), 5.37 (d, J = 8.2 Hz, 1H, NH), 7.18–7.31 (m, 9 H_{arom}), 7.53 bs, 1H, NH). FAB-HRMS calcd. for $C_{27}H_{34}N_5O_4$, 492.2611; found, 492.2586.

5.2. Biological evaluation

Z-Pro-L-Prolinal-dimethylacetal, Boc-Asn-Phe-Pro aldehyde and Z-Pro-Pro-OH were purchased from Bachem. The fluorogenic substrate *N*-Suc-Gly-Pro-Leu-Gly-Pro-AMC was from Sigma and DFP from ICN. Fluorescence was measured in a Hitachi 2000 spectrofluorimeter at excitation and emission wavelengths of 380 and 440 nm, respectively.

5.2.1. Enzyme assay and determination of IC_{50} values

Enzymatic reactions were carried out in 25 mM Tris-HCl, pH 7.5, 125 mM NaCl, at 24 °C. Substrate and inhibitor stock solutions were prepared in DMSO, stored at –20 °C and diluted in the reaction buffer just prior to assays. IC_{50} values were determined after pre-incubation for 10 min with 30 μL of purified enzyme (1–5 nM solution in the reaction), with 30 μL of inhibitor solution at a concentration three times the final concentration desired and then by addition of 30 μL of a 100 μM substrate solution. The reaction was stopped 15 min later by addition of ethanol (1 mL) and the fluorescence measured. The final inhibitor concentrations used ranged from 1 nM to 100 μM. The percentage of inhibition was calculated by comparison with the fluorescence measured in samples containing reaction buffer instead of inhibitor. The IC_{50} value was estimated from the inhibition versus inhibitor concentration curve. Data presented are the mean ± standard deviation of at least three independent experiments.

5.2.2. Type of inhibition and determination of inhibition constants

Hydrolyses were standardized using a 0.05–0.5 μM AMC calibration. The enzyme (300 μL, 0.1–0.5 nM solution in reaction buffer) was mixed for 5 min with 300 μL of substrate prepared in the same buffer, before adding 3 μL of the inhibitor solution or the equivalent amount of DMSO. The hydrolysis kinetics were recorded up to 45 min and slopes expressed in nmol of AMC released per min using standard curves. The final substrate concentrations used ranged from 3.3–166 μM, and the inhibitor concentrations from 20 nM to 1 μM.

5.2.3. Reversibility of the inhibition

30 μL of inhibitor (20 μM) were incubated with 30 μL of purified enzyme for 2 h at 37 °C. The sample was then dialysed by ultrafiltration through five successive steps of dilution in 2 mL reaction buffer, and of concentration using an Amicon unit (cut off 30 kDa). After a last concentration to 100 μL, the enzymatic activity was measured and compared to that measured in samples incubated without inhibitor and processed in a same way.

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References

[1] Tardieux I., Webster P., Ravesloot J., Boron W., Lunn J.A., Heuser J.E., Andrews N.W., Cell 71 (1992) 1117–1130.

[2] Santana J.M., Grellier P., Rodier M.H., Schrével J., Teixeira A.R.L., Biochem. Biophys. Res. Commun. 187 (1992) 1466–1473.

[3] Burleigh B.A., Caler E.V., Webster P., Andrews N.W.J., Cell Biol. 136 (1997) 609–620.

[4] Garcia M.P., Nobrega O.T., Teixeira A.R.L., Sousa M.V., Santana J.M., Mol. Biochem. Parasitol. 91 (1998) 263–272.

[5] Santana J.M., Grellier P., Schrével J., Teixeira A.R.L., Biochem. J. 324 (1997) 129–137.

[6] Nham S., Weinreb S.M., Tetrahedron Lett. 22 (1981) 3815–3818.

[7] Sibi M.P., Org. Prep. Proced. Int. 25 (1993) 15–40.

[8] Roux F., Maugras I., Poncet J., Niel G., Jouin P., Tetrahedron 50 (1994) 5345–5360.

[9] Palmer J.T., Rasnick D., Klaus J.L., Brömme D., J. Med. Chem. 38 (1995) 3193–3196.

[10] Fehrentz J.A., Castro B., Synthesis (1983) 676–678.

[11] Costanzo M.J., Maryanoff B.E., Hecker L.R., Schott M.R., Yabut S.C., Zhang H.C. et al., J. Med. Chem. 39 (1996) 3039–3043.

[12] Reetz M.T., Drewes M.W., Lennick A., Schmitz A., Holdgrün X., Tetrahedron Asym. 1 (1990) 375–378.

[13] Nakajima N., Ubukata M., Tetrahedron Lett. 38 (1997) 2099–2102.

[14] Xue C.B., Degrado W.F., Tetrahedron Lett. 36 (1995) 55–58.

[15] Campagna F., Carotti A., Casini G., Tetrahedron Lett. 21 (1977) 1813–1816.

[16] Li J., Wilk E., Wilk S., J. Neurochem. 66 (1996) 2105–2112.

[17] Holzapfel C.W., Pettit G.R., J. Org. Chem. 50 (1985) 2323–2327.

[18] Maekawa K., Ohtani J., Agric. Biol. Chem. 41 (1977) 811–818.

[19] Henderson P.J.F., Biochem J. 127 (1972) 321–333.

[20] Salvesen G., Nagase H., Bond J.S., Beynon R.J. (Eds.), Proteolytic Enzymes: a Practical Approach, IRL Press, Oxford, 1989, pp. 83–104.

[21] Cornish-Bowden A., Principles of Enzyme Kinetics, Butterworths, London, 1976.

[22] Yoshimoto T., Orlowski R.C., Walter R., Biochemistry 16 (1977) 2942–2948.

[23] Yoshimoto T., Sattar A.K.M.A., Hirose W., Tsuru D., J. Biochem. (Tokyo) 104 (1988) 622–627.

[24] Barret A.J., in: Barret A.J. (Ed.), Methods in Enzymology, Proteolytic Enzymes: Serine and Cysteine Peptidases Vol. 244, Academic Press, San Diego, 1994, pp. 1–15.

[25] Moriyama A., Sasaki M., J. Biochem. (Tokyo) 94 (1983) 1387–1397.

[26] Yoshimoto T., Sattar A.K.M.A., Hirose W., Tsuru D., Biochim. Biophys. Acta 916 (1987) 29–37.

[27] Sattar A.K.M.A., Yamamoto N., Yoshimoto T., Tsuru D., J. Biochem. (Tokyo) 107 (1990) 256–261.

[28] Polgar L., in: Barret A.J. (Ed.), Methods in Enzymology, Proteolytic Enzymes: Serine and Cysteine Peptidases Vol. 244, Academic Press, San Diego, 1994, pp. 188–200.

[29] Liu S., Hanzlik R.P., J. Med. Chem. 35 (1992) 1067–1075.

[30] Demuth H.U., Schlenzig D., Schierhorn A., Grosche G., Chapot-Chartier M.P., Gripon J.C., FEBS Lett. 320 (1993) 23–27.

[31] Stone R., Rennex D., Wikstrom P., Shaw E., Hofsteenge J., Biochem. J. 283 (1992) 871–876.

[32] Edwards P.D., Meyer E.F., Vijayalakshmi J., Tuthill P.A., Andisik D.A., Gomes B., Strimpler A., J. Am. Chem. Soc. 114 (1992) 1854–1863.

[33] Tsutsumi S., Okonogi T., Shibahara S., Ohuchi S., Hatsushiba E., Patchett A.A., Christensen B.J., J. Med. Chem. 37 (1994) 3492–3502.

[34] Li J., Wilk E., Wilk S., Arch. Biochem. Biophys. 323 (1995) 148–154.

[35] Chen J.J., Zhang Y., Hammond S., Dewdney N., Ho T., Lin X., Browner M.F., Castelhano A.L., Bioorg. Med. Chem. Lett. 6 (1996) 1601–1606.

[36] Fülöp V., Böcskei Z., Polgar L., Cell 94 (1998) 161–170.

[37] Wilk S., Orlowski M., J. Neurochem. 41 (1983) 69–75.

[38] Augustyns K., Borloo M., Belyaev A., Rajan P., Goossens F., Hendriks D., Scharpé S., Haemers A., Bio. Med. Chem. Lett. 5 (1995) 1265–1270.

[39] Vendeville S., Buisine E., Williard X., Schrevel J., Grellier P., Santana J., Sergheraert C., Chem. Pharm. Bull. 47 (1999) 194–198.

[40] Vendeville S., Bourel L., Davioud-Charvet E., Grellier P., Deprez B., Bio. Med. Chem. Lett. 9 (1999) 437–442.