Identification of Inhibitors of an 80 kDa Protease from *Trypanosoma cruzi* through the Screening of a Combinatorial Peptide Library

Sandrine VENDEVILLE,[a] Eric BUISINE,[a] Xavier WILLIARD,[a] Joseph SCHREVEL,[b] Philippe GRELLIER,[b] Jaime SANTANA,[c] and Christian SERGHERAERT*,[a]

Institut de Biologie de Lille-Institut Pasteur de Lille, URA CNRS 1309, Faculté de Pharmacie, Université de Lille II,[a] 1 rue Calmette, BP 447, 59021 Lille Cedex, France, Museum National d'Histoire Naturelle, Laboratoire de Biologie Parasitaire et Chimiothérapie, EP CNRS 1790,[b] 61 rue Buffon, 75005 Paris, France, and Laboratorio Multidisciplinar de Pesquisa em Doença de Chagas, Departamentos de Biologia Celular e de Patologia,[c] Universidade de Brasilia, CP 04536, 70919–970, Brasilia, Brazil. Received August 19, 1998 ; accepted November 12, 1998

Two orthogonal peptide combinatorial libraries were screened to discover inhibitors of Tc80 protease, a novel target from *Trypanosoma cruzi* involved in host cell invasion. These libraries were composed of 15625 structurally diversified tripeptides, partitioned in 125 mixtures. The screening led to a low micromolar inhibitor which was actually an HF cleavage by-product H-Ipe-D-Tic-D-Glu(S-paratolyl)-OH. IC_{50} values of several analogous molecules of this hit were determined and are discussed. For the best compounds, conformational analysis revealed a high degree of similarity in shape with a potent prolylendopeptidase inhibitor, SUAM-1221.

Key words Chagas disease; *Trypanosoma cruzi*; protease inhibitor; combinatorial chemistry; tripeptide; tetrahydroisoquinoleic acid

The flagellated protozoan *Trypanosoma cruzi* is the etiological agent of Chagas disease, a chronic, debilitating, incurable illness that is highly prevalent in Latin America.[1] *T. cruzi* trypomastigote forms use the bloodstream to reach and infect a broad range of tissues and cells. To have access to different cell types, the parasite must cross an intricate network of fibrous proteins named the extracellular matrix.[2] In respect of these features of the parasitic cycle, Santana *et al.* postulated that *T. cruzi* may express specific proteases that could facilitate parasitic migration through the host matrix. They characterized a *T. cruzi*-secreted 80 kDa protease (Tc80), that specifically hydrolyses purified human collagens type I and IV, and native collagen type I in rat mesentery at neutral pH.[3] Thus, Tc80 may facilitate host cell infection by degradation of the extracellular matrix collagens. Tc80 also hydrolyses the fluorogenic substrate N-succinyl-Gly–Pro–Leu–Gly–Pro-AMC, derived from repetitive sequences of human collagen type I, between proline and AMC, suggesting that Tc80 could be a member of the prolylendopeptidase family. However, belonging to a specific class of proteases: serine, cysteine, aspartic or metalloproteases, remains a debatable question for this enzyme because of the variety of information yielded by the main irreversible protease inhibitors.[3]

In this paper, we report the use of two orthogonal peptide combinatorial libraries to quickly obtain inhibitors of Tc80. These orthogonal self-deciphering libraries, recently described by our group, can offer the advantage of an internal validation, as any positive result detected in one library must be confirmed by a positive result in the orthogonal library.[4] Each library corresponds to 15625 tripeptides, synthesized from 23 D-amino acids and 2 non-chiral amino acids, divided in 125 mixtures. To synthesize a library, the 25 amino acids were partitioned into five groups of five amino acids, each named A_1—A_5 for library A and B_1—B_5 for library B (Fig. 1). For each group, amino acids were especially selected in order to maximize the structural diversity. For the synthesis of library A, each group (A_1 to A_5), was coupled to a chloromethylated Merrifield resin Polym-CH_2Cl, and the five mixtures of aminoacyl resins were split into five parts each and coupled with the mixtures of amino acids, leading, after deprotection and cleavage from the resin, to 125 sub-libraries in a soluble form. Library B was prepared according to the same procedure, except that in this case, the orthogonal groups of amino acids (B_1 to B_5), were used.

	A_1	A_2	A_3	A_4	A_5
B_1	D-Leucine	D-Proline	D-Serine	(4-Nitro)-D-phenylalanine	D-Isoglutamic acid
B_2	D-Arginine	D-Isoleucine	Glycine	D-Threonine	D-Isoglutamine
B_3	D-Glutamine	D-Tyrosine	D-Valine	D-ε-Nicotinoyl lysine	D-Histidine
B_4	D-Tryptophane	D-Asparagine	D-Glutamic acid	D-Methionine sulfoxide	D-Alanine
B_5	Isonipecotic acid	D-Lysine	D-Tetrahydro-isoquinoleic acid	D-Aspartic acid	D-Phenylalanine

Fig. 1. Repartition of the 25 Amino Acids Used for the Synthesis of the Combinatorial Libraries

* To whom correspondence should be addressed.

Results

Both libraries were examined according to the following method. The first library (A), was studied at a concentration of 333 μM for each of the 125 mixtures of 125 tripeptides. Unfortunately, at this and lower concentrations, inhibition values were almost identical for each mixture, rendering the results unusable. In the second library (B), at 333 μM, 11 mixtures displayed an inhibition greater than 50%, with the best being, $X_{B5}X_{B5}X_{B1}$, with 80% inhibition of the protease activity. A deconvolution method, known as "positional scanning,"[5,6] had therefore to be used to identify the tripeptide(s) responsible for activity. New syntheses were carried out under the conditions previously described for libraries A and B. The inhibiting potency of the different mixtures was evaluated by measuring IC_{50} values in the presence of 11 μM succinyl-Gly–Pro–Leu–Gly–Pro-AMC as substrate.

The first series of five syntheses $X_{B5}X_{B5}O_{B1}$ (X_{B5}=mixture of the five amino acids of B_5; O_{B1}=only one of the five amino acids of B_1 group, comprising D-Leu, D-Pro, D-Ser, 4-nitro-D-Phe and D-isoGlu), led us to define D-Glu as the C-terminal residue for the best mixture of 25 tripeptides displaying an IC_{50} of 0.65 μM (the mixture with 4-nitro-D-Phe was active to a lesser degree). The second series of five syntheses $X_{B5}O_{B5}X_{B1}$ gave us D-Tic (tetrahydroisoquinoleic acid) as the intermediate residue for the best mixture of 25 tripeptides displaying an IC_{50} of 0.8 μM, while for the third series $O_{B5}X_{B5}X_{B1}$, there remained little to choose between Ipe (isonipecotic acid), D-Tic, and D-Asp for the best N-terminal residue (IC_{50} values around 4 μM). This approximate activity led us to synthesize and to test the three corresponding tripeptides: H-Ipe-D-Tic-D-Glu-OH, H-D-Tic-D-Tic-D-Glu-OH, H-D-Asp-D-Tic-D-Glu-OH, the first one displaying the best inhibition with an IC_{50} of 3 μM before purification (IC_{50} higher than 30 μM for the two others).

The purification and analysis of the crude product, expected to be H-Ipe-D-Tic-D-Glu(OH)-OH, revealed that the major constituent was in fact, the HF cleavage by-product H-Ipe-D-Tic-D-Glu(S-paratolyl)-OH **1** with a smaller quantity of the corresponding paracresol ester **2**. Numerous side reactions with glutamic acid have already been described as occuring during HF/scavenger cleavage step.[5,7—9] Esterification of glutamic acid by scavengers (parathiocresol or paracresol), is explained by nucleophilic attack by the sulfur or oxygen atom upon the activated γ carboxyl and here, the higher nucleophilic character of the sulfur atom justifies the greater formation of thiocresol derivative. Cleavage of the H-Ipe-D-Tic-D-Glu(OH)-O-resin without scavengers, mainly yielded H-Ipe-D-Tic-D-Glu(OH)-OH (**3**) which when isolated, proved to be inactive.

The presence of an aromatic ring in the C-terminal position seemed therefore critical for inhibition, all the more so, since among the XXO_{B1} mixtures, only those corresponding to 4-nitro-D-Phe and D-Glu(S-paratolyl), were active. The sensitivity of aromatic thioesters to hydrolysis led us first to prepare their esters and amide bioisosteres with paracresol or benzylic alcohol and paratoluidine respectively (compounds **2**, **4**, **5**). New products turned out to be as active (IC_{50} values around 12 μM), as the reference tripeptide **1** (Table 1). However, when paratoluidine was positioned on the glutamyl α carbonyl (compound **6**), inhibition decreased, suggesting that a minimum spacer length is required for inhibition. Modification of the N-terminal extremity was also studied and the isonipecotic residue was replaced by a cyclohexyl (compound **7**), or a phenyl ring (compound **8**), to determine respectively, the influence of a basic site and of a π aromatic moiety in this position. Both analogues were less active than the reference tripeptide **5**. Finally, the stereochemistry of the two chiral residues was studied with the synthesis of derivatives **9** and **10** of the L series. Contrary to Glu stereochemistry which seemed not to be essential for inhibition (compound **10** displayed an IC_{50} of 32 μM), Tic stereochemistry was found to be critical for inhibition as no activity was observed with compound **9** at concentrations below 80 μM.

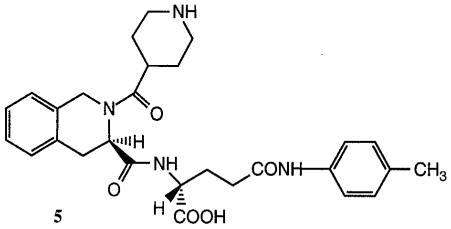
Table 1. Inhibitory Activities of Compounds **1**—**10** towards Tc80 Protease

No.	Compound	IC_{50} (μM)
1	H-Ipe-D-Tic-D-Glu(S-paratolyl)-OH	12
2	H-Ipe-D-Tic-D-Glu(O-paratolyl)-OH	13
3	H-Ipe-D-Tic-D-Glu(OH)-OH	>80
4	H-Ipe-D-Tic-D-Glu(OBzl)-OH	—[a]
5	H-Ipe-D-Tic-D-Glu(NH-paratolyl)-OH	12
6	H-Ipe-D-Tic-D-Glu(OH)-NH-paratolyl	30
7	Cyclohexylcarbonyl-D-Tic-D-Glu(NH-paratolyl)-OH	39
8	Phenylcarbonyl-D-Tic-D-Glu(NH-paratolyl)-OH	27
9	H-Ipe-L-Tic-D-Glu(NH-paratolyl)-OH	>80
10	H-Ipe-D-Tic-L-Glu(NH-paratolyl)-OH	32

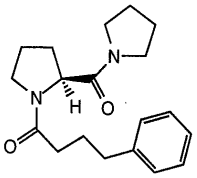
a) IC_{50} not determined because of the insolubility of the compound.

Recognition of the Tic residue by Tc80 and Tic/Proline analogy, in addition to the already reported substrate specificity and molecular weight of the enzyme,[3] led us to assume that our protease could be a prolylendopeptidase. Moreover, the conformational analysis of H-Ipe-D-Tic-D-Glu(NH-paratolyl)-OH **5** and phenylpropylcarbonyl-L-prolyl-pyrrolidine (the latter known as SUAM-1221),[10—12] a potent prolylendopeptidase inhibitor, permitted the identification of low-energy conformers adopting a similar U-shape already reported for SUAM-1221.[13] The two conformers superimposed in Fig. 2 are in the *cis* form with respect to the N-terminal amide linkage. Although this form was found to be energetically favoured over the *trans*, the small energy difference; 0.5 kcal/mol and 1.7 kcal/mol for **5** and SUAM-1221 respectively, does not aid in the deduction of which is the biologically active one. Furthermore, NMR analysis of both compounds revealed that two forms exist in solution (for SUAM-1221, see reference 14, for compound **5**, see experimental data). The D-Tic ring of **5** could thus mimic the L-Pro ring of SUAM-1221, while the C-terminal D-Glu(NH-paratolyl)-OH, could overlap with the N-terminal propylphenyl moiety and the N-terminal Ipe moiety with the C-terminal pyrrolidine ring (these two molecules can be regarded as "retro-inverso analogues").[14] The superimposition shows that compound **5** matches the pharmacophore model of SUAM-1221.[13] This pharmacophore is characterized by three spatially close anchorage points: two hydrogen-bond acceptors (the N- and C-terminal carbonyl groups), and a hydrophobic moiety (phenyl ring), that could be responsible for the potency of numerous prolylendopeptidase inhibitors, as previously reported.

In conclusion, the use of a combinatorial library of tripeptides allowed us both to quickly find inhibitors of Tc80 from *T. cruzi* and to identify an original tripeptide structure con-



5



SUAM 1221

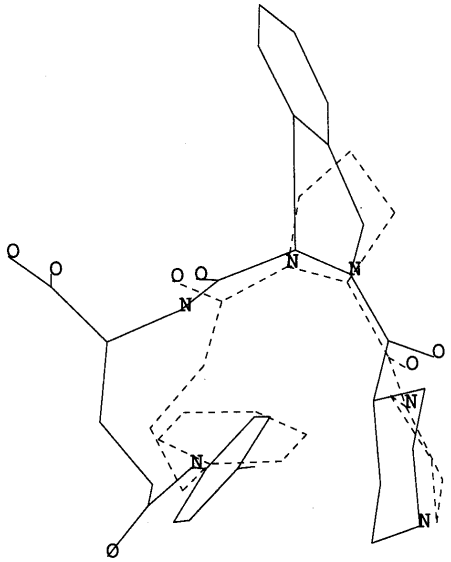


Fig. 2. Best-overlap Obtained for H-Ipe-D-Tic-D-Glu(NHparatolyl)-OH **5** and SUAM-1221

The latter compound is shown in dashed lines. The superimposition was performed using the coordinates of ring centers (saturated six-membered ring of D-Tic residue and prolyl ring of L-Pro residue, the phenyl moieties of paratoluidine and phenylpropyl sidechains, isonipecotic and pyrrolidine ring) and amide oxygen atoms of the two molecules (C-terminal amide oxygen of D-Tic residue, N-terminal amide oxygen of L-Pro residue, N-terminal amide oxygen of D-Tic residue, C-terminal amide oxygen of L-Pro residue). The overall root mean square deviation (rmsd), is ≈0.58 Å.

taining a non-natural amino acid, Tic, as the central residue. The Tic/proline analogy and conformational similarities between one of our most potent inhibitors and a prolyl endopeptidase (PEP), inhibitor named SUAM-1221 enabled us to classify this parasitic protease in the proline-specific oligopeptidase family. Furthermore, the synthesis of specific inhibitors of Tc80 based upon the Tic residue could constitute new tools to evaluate the role of this enzyme in the biology of *T. cruzi*. A study of the specificity against other prolyl endopeptidases will be presented in a further paper.

Experimental

Amino-acid derivatives were purchased from Bachem. Analytical reversed-phase high performance liquid chromatography (RP-HPLC), was performed on a Shimadzu liquid chromatograph using 25×200 cm stainless steel columns packed with C_{18} derivatized silica (7 μm, 300 A). Chromatograms were obtained at flow rates of 1 ml/min using a linear gradient of (A) and (B) (0—100% B, 30 min), with UV detection at 254 nm. Preparative RP-HPLC was carried out on a Gilson system in combination with a chart recorder and a UV detector. The following solvent systems were used: solvent A: 0.05% trifluoroacetic acid (TFA) in H_2O; solvent B: 0.05% TFA, 20% H_2O, 80% CH_3CN. Mass spectra were obtained on a time-of-flight plasma desorption mass spectrometer (TOF-PDMS), using a californium source. NMR spectra were recorded on a Brucker DRX 300 MHz spectrometer; coupling constants are expressed in Hz and chemical shifts in ppm relative to TMS as an internal standard. Abbreviations were used as follows: Tic=1,2,3,4-tetrahydroisoquinoleic acid; Ipe=isonipecotic acid; DCC=*N,N'*-dicyclohexylcarbodiimide; DCU=*N,N'*-dicyclohexylurea; HOBt=1-hydroxybenzotriazole; HBTU=*O*-(1*H*-benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate; HF=hydrogen fluoride; TFA=trifluoroacetic acid; DIEA=*N,N'*-diisopropylethylamine; DCM =methylene chloride; DMF=*N,N'*-dimethylformamide; AMC=7-amino-4-methylcoumarin.

Preparation of a tBoc-Aminoacyl-resin tBoc-D-Glu(OBzl)-OH (1.95 g, 6 mmol), was added to a stirred solution of EtOH (30 ml), containing 3 ml of 1 M Cs_2CO_3. Solvent was then evaporated at 40 °C under reduced pressure. A mixture of toluene/ethanol (1/1) was added three times, to the residue and evaporated to completely eliminate residual water. The resulting oily cesium salt was solubilized in 25 ml DMF and Merrifield resin (5.72 g, 4 mmol, 0.70 mmol/g), was added. The mixture was gently stirred at 50 °C for 24 h. The resin was then filtered and washed with 50 ml DMF (3 times), DMF/H_2O 1/1 (3 times), DMF/EtOH 1/1 (3 times), EtOH (3 times), MeOH (3 times), DCM (3 times), and dried at 50 °C overnight: loading: 0.636 mmol/g.

General Procedure for the Synthesis of the Two Orthogonal Libraries and Deconvolution Mixtures tBoc-stategy with HBTU/HOBt activated esters was employed using a chloromethylated resin. Benzyl-based protections were used for side-chains. The tBoc-protected aminoacids were esterified to the resin as described above. Removal of the tBoc-protecting group was achieved in TFA/DCM 1/1 for 30 min. After a neutralization step in DIEA/DCM 5/95, amino acid coupling was achieved by *in situ* activation of 1.1 equivalent of tBoc-amino acid and 1.1 equivalent of HBTU and HOBt in DMF for 2 h. A second coupling step was then performed to lead the reaction to completion which was monitored by the Kaiser ninhydrin test. The tripeptides were removed from the resin and side-chains deprotected in anhydrous HF containing parathiocresol (25 mg/ml of HF), and paracresol (75 mg/ml of HF), at 0 °C for 1.5 h. The resulting mixtures of peptides were precipitated with a mixture diethylether/pentane, dissolved in TFA, reprecipitated and lyophilized from acetic acid.

Preparation of H-Ipe-D-Tic-D-Glu(OH)-OH (3), H-D-Tic-D-Tic-D-Glu-(OH)-OH and H-D-Asp-D-Tic-D-Glu(OH)-OH Synthesis and cleavage of these three compounds were performed on 0.2 mmol (127 mg), of tBoc-D-Glu-OBzl(O-resin), for each tripeptide in the same manner as reported for the libraries. HPLC analysis of the crude tripeptides show a purity of 30—70% and no purification of these products was attempted prior to bioassay. For the most active crude tripeptide, compound **3** and side-products **1** and **2** were isolated by preparative RP-HPLC using a linear gradient of (A) and (B) (0—100% B, 1 h). They were characterized by mass spectroscopy and NMR.

(1) H-Ipe-D-Tic-D-Glu(S-paratolyl)-OH: Yield, 18%. TOF-PDMS (*m/z*)= 523.5 (M^+). HPLC (t_R=19.460 min). ^{1}H-NMR (DMSO-d_6): 1.50—2.30 (m, 11H), 2.68—3.25 (m, 7H), 3.75—3.90 (m, 1H, $CH_{D\text{-}Glu}$), 4.47+4.64 (d, 1H, *J*=16 Hz, $H_{1\text{-}Tic}$, *cis*+*trans*: 50/50), 4.53+4.78 (d, 1H, *J*=16 Hz, $H_{1\text{-}Tic}$), 4.80+4.86 (m, 1H, $H_{3\text{-}Tic}$), 7.05—7.25 (m, 8H), 7.62+7.78 (m, 1H, $NH_{D\text{-}Glu}$).

(2) H-Ipe-D-Tic-D-Glu(O-paratolyl)-OH: Yield, 7%. TOF-PDMS (*m/z*)= 507.3 (M^+). HPLC (t_R=18.055 min). ^{1}H-NMR (DMSO-d_6): 1.70—2.30 (m, 11H), 2.72—3.38 (m, 7H), 4.00—4.15 (m, 1H, $CH_{D\text{-}Glu}$), 4.53+4.58 (d, 1H, *J*=16 Hz, $H_{1\text{-}Tic}$, *cis*+*trans*: 40/60), 4.69+4.83 (d, 1H, *J*=16 Hz, $H_{1\text{-}Tic}$), 4.85+4.91 (m, 1H, $H_{3\text{-}Tic}$), 6.93—6.98 (m, 2H, paracresol), 7.16—7.28 (m, 6H), 7.65+7.80 (m, 1H, $NH_{D\text{-}Glu}$).

(3) H-Ipe-D-Tic-D-Glu(OH)-OH: Yield, 15%. TOF-PDMS (*m/z*)=417 (M^+). HPLC (t_R=12.610 min). ^{1}H-NMR (DMSO-d_6): 1.61—2.10 (m, 8H), 2.74—3.53 (m, 7H), 4.46—4.95 (m, 4H), 7.17—7.30 (m, 4H), 7.85+8.00 (d, 1H, *J*=8.5 Hz, $NH_{D\text{-}Glu}$, *cis*+*trans*: 50/50), 8.27+8.45 (broad, 1H, NH_{Ipe}), 12.20 (broad, 2H, COOH).

Direct Preparation of H-Ipe-D-Tic-D-Glu(OH)-OH (3) The synthesis of this compound was performed in the same way as above from tBoc-D-Glu-OBzl(O-resin), except that no scavenger was used during the HF-cleavage step. Compound **3** was obtained in a pure form, with a yield of 42%.

Preparation of Starting Materials for Synthesizing Compounds 4—10. Fmoc-D-Glu(NH-paratolyl)-OtBu To 600 mg (1.410 mmol), of Fmoc-D-Glu(OH)-OtBu in DCM (20 ml), were added paratoluidine (151 mg, 1.410 mmol), HOBt (190 mg, 1.410 mmol), in DMF (2 ml). DCC (291 mg, 1.410

mmol) was then added and the reaction mixture was stirred at room temperature overnight. DCU was filtered, and solvents evaporated. The residue was taken up by EtOAc and washed with aqueous solutions of $NaHCO_3$, citric acid and brine. After drying over $MgSO_4$ and evaporation, the solid was purified by preparative TLC (DCM–MeOH, 95 : 5) to yield 650 mg (90%), of the desired product. TOF-PDMS (m/z)=514.5 (M^+), 537.3 ($M+Na^+$). HPLC (t_R=28.670 min). ^{1}H-NMR (DMSO-d_6): 1.41 (s, 9H, Boc), 1.77—1.90 (m, 1H, C$\underline{H}_2$CH$_2$CO), 2.00—2.08 (m, 1H, C$\underline{H}_2$CH$_2$CO), 2.24 (s, 3H, CH$_3$), 2.41 (t, 2H, J=7.43 Hz, CH$_2$C$\underline{H}_2$CO), 3.91—3.98 (m, 1H, CH$_{D\text{-}Glu}$), 4.20—4.34 (m, 3H, H$_{Fmoc}$), 7.09 (d, 2H, J=8.29 Hz, H$_{paratoluidine}$), 7.34 (t, 2H, J=7.42 Hz, H$_{Fmoc}$), 7.43 (t, 2H, J=7.42 Hz, H$_{Fmoc}$), 7.46 (d, 2H, J=8.29 Hz, H$_{paratoluidine}$), 7.73—7.75 (m, 3H), 7.91 (d, 2H, J=7.42 Hz, H$_{Fmoc}$), 9.83 (s, 1H, NH$_{paratoluidine}$).

Fmoc-D-Glu(OtBu)-NH-paratolyl: Yield, 87%. TOF-PDMS (m/z)=514.1 (M^+). HPLC (t_R=28.203 min). ^{1}H-NMR (DMSO-d_6): 1.41 (s, 9H, Boc), 1.84—2.00 (m, 2H, C$\underline{H}_2$CH$_2$COOH), 2.25 (s, 3H, CH$_3$), 2.30—2.33 (m, 2H, CH$_2$C$\underline{H}_2$COOH), 4.02—4.30 (m, 4H), 7.11 (d, 2H, J=8.25 Hz, H$_{paratoluidine}$), 7.34 (t, 2H, J=7.45 Hz, H$_{Fmoc}$), 7.43 (t, 2H, J=7.45 Hz, H$_{Fmoc}$), 7.49 (d, 2H, J=8.25 Hz, H$_{paratoluidine}$), 7.70—7.75 (m, 3H), 7.91 (d, 2H, J=7.45 Hz, H$_{paratoluidine}$), 9.95 (s, 1H, NH$_{paratoluidine}$).

Fmoc-D-Glu(NH-paratolyl)-OH: 650 mg (1.264 mmol) of Fmoc-D-Glu-(NH-paratolyl)-OtBu was deprotected in a solution of 50% TFA in DCM (20 ml) at room temperature for 2 h. Excess acid and solvent were evaporated under reduced pressure. The residue was re-evaporated twice from petroleum ether and crystallized from an acetone–petroleum ether mixture to give 578 mg (100%) of a white solid. TOF-PDMS (m/z)=458 (M^+). HPLC (t_R=24.700 min). ^{1}H-NMR (DMSO-d_6): 1.82—1.89 (m, 1H, C$\underline{H}_2$CH$_2$CO), 2.06—2.13 (m, 1H, C$\underline{H}_2$CH$_2$CO), 2.25 (s, 3H, CH3), 2.42 (t, 2H, J=7.70 Hz, CH$_2$C$\underline{H}_2$CO), 3.97—4.05 (m, 4H), 7.09 (d, 2H, J=8.30 Hz, H$_{paratoluidine}$), 7.34 (t, 2H, J=7.32 Hz, H$_{Fmoc}$), 7.43 (t, 2H, J=7.32 Hz, H$_{Fmoc}$), 7.47 (d, 2H, J=8.30 Hz, H$_{paratoluidine}$), 7.70—7.76 (m, 3H), 7.91 (d, 2H, J=7.32 Hz, H$_{Fmoc}$), 9.83 (s, 1H, NH$_{paratoluidine}$).

Fmoc-D-Glu(OH)-NH-paratolyl: Yield, 99%. TOF-PDMS (m/z)=458.6 (M^+). HPLC (t_R=24.191 min). ^{1}H-NMR (DMSO-d_6): 1.85—1.99 (m, 2H, C$\underline{H}_2$CH$_2$COOH), 2.26 (s, 3H, CH$_3$), 2.29—2.36 (m, 2H, CH$_2$C$\underline{H}_2$COOH), 4.13—4.31 (m, 4H), 7.12 (d, 2H, J=8.22 Hz, H$_{paratoluidine}$), 7.34 (t, 2H, J=7.44 Hz, H$_{Fmoc}$), 7.43 (t, 2H, J=7.44 Hz, H$_{Fmoc}$), 7.50 (d, 2H, J=8.22 Hz, H$_{paratoluidine}$), 7.69—7.77 (m, 3H), 7.91 (d, 2H, J=7.44 Hz, H$_{Fmoc}$), 9.95 (s, 1H, NH$_{paratoluidine}$).

Fmoc-D-Glu(OBzl)-OH: 1.340 g (5.650 mmol) of H-D-Glu(OBzl)-OH was dissolved in a 10% solution of Na_2CO_3 in water (15 ml) and dioxane (9 ml). 1.460 g (5.650 mmol) of 9-fluorenylmethyl chlorocarbonate was then added at ice-water bath temperature and the mixture was stirred overnight at room temperature. The reaction mixture was poured into water (300 ml) and extracted with ether (100 ml). The aqueous solution was cooled in an ice-bath, acidified under vigorous stirring with concentrated hydrochloric acid to Congo and extracted with DCM (2×50 ml). After drying over $MgSO_4$ and evaporation, the product was purified by flash chromatography (EtOAc) to yield 1.167 g (45%) of a white solid. TOF-PDMS (m/z)=459 (M^+). HPLC (t_R=26.325 min). ^{1}H-NMR (DMSO-d_6): 1.75—2.08 (m, 2H, C$\underline{H}_2$CH$_2$CO), 2.26—2.40 (m, 2H, CH$_2$C$\underline{H}_2$CO), 3.80 (m, 1H, CH$_{D\text{-}Glu}$), 4.18—4.37 (m, 3H), 5.06 (s, 2H, C$\underline{H}_2$C$_6$H$_5$), 7.28—7.42 (m, 10H), 7.68 (d, 2H, J=7.35 Hz, H$_{Fmoc}$), 7.88 (d, 2H, J=7.35 Hz, H$_{Fmoc}$).

General Procedure for the Syntheses of Compounds 4—10 Solid-phase synthesis on the 2-chlorotritylchloride resin using a Fmoc-strategy with HBTU-activated esters was employed. Coupling to the resin (1 eq, 0.97 mmol/g) was achieved using 0.6 eq of carboxylic acid (Fmoc-D-Glu(NH-paratolyl)-OH, Fmoc-D-Glu(OH)-NH-paratolyl, Fmoc-L-Glu(NH-paratolyl)-OH or Fmoc-D-Glu(OBzl)-OH), and 4 eq of DIEA in dry DCM for 30 min. The Fmoc-protecting group was removed by 20% piperidine/DMF for 15 min. Amino acid coupling was then achieved by *in situ* activation of 1.25 eq of carboxylic acid, 1.25 eq of HBTU and 2.5 eq of DIEA in a mixture of DCM and DMF for 1 h. The reaction was monitored by the Kaiser or Christensen tests. In the case of incomplete reaction, the coupling step was repeated. Final cleavage of the desired molecule from the resin was achieved by 1% TFA in DCM for 10 min. No purification step was needed.

(4) H-Ipe-D-Tic-D-Glu(OBzl)-OH: Yield, 40%. TOF-PDMS (m/z)=507.5 (M^+). HPLC (t_R=16.750 min). ^{1}H-NMR (DMSO-d_6): 1.61—2.29 (m, 8H), 2.52—3.34 (m, 7H), 4.12 (m, 1H, CH$_{D\text{-}Glu}$), 4.47 (m, 1H, H$_{1\text{-}Tic}$), 4.63 (m, 1H, H$_{1\text{-}Tic}$), 4.80—4.87 (m, 1H, H$_{3\text{-}Tic}$), 5.08 (s, 2H, C$\underline{H}_2$C$_6$H$_5$), 7.05—7.45 (m, 9H, aromatic), 7.97+8.02 (d, 1H, J=7.09 Hz, NH$_{D\text{-}Glu}$, *cis*+*trans*: 45/55), 8.30 (broad, 1H, NH$_{Ipe}$), 12.60 (broad, 1H, COOH).

(5) H-Ipe-D-Tic-D-Glu(NH-paratolyl)-OH : Yield, 60%. TOF-PDMS (m/z) =506.8 (M^+). HPLC (t_R=15.535 min). ^{1}H-NMR (DMSO-d_6): 1.55—2.28 (m, 11H), 2.59—3.38 (m, 7H), 4.12—4.16 (m, 1H, CH$_{D\text{-}Glu}$), 4.52+4.62 (d, 1H, J=16.4 Hz, H$_{1\text{-}Tic}$, *cis*+*trans*: 50/50), 4.68+4.84 (d, 1H, J=16.4 Hz, H$_{1\text{-}Tic}$, *cis*+*trans*), 4.86+4.91 (m, 1H, H$_{3\text{-}Tic}$, *cis*+*trans*), 7.11 (d, 2H, J=7.95 Hz, H$_{paratoluidine}$), 7.15—7.30 (m, 4H, H$_{Tic}$), 7.47 (d, 2H, J=7.95 Hz, H$_{paratoluidine}$), 8.14+8.29 (d, 1H, J=7.80 Hz, NH$_{D\text{-}Glu}$, *cis*+*trans*), 8.34+8.60 (broad, 1H, NH$_{Ipe}$, *cis*+*trans*), 9.67+9.83 (broad, 1H, NH$_{paratoluidine}$, *cis* +*trans*), 12.60 (broad, 1H, COOH).

(6) H-Ipe-D-Tic-D-Glu(OH)-NH-paratolyl: Yield, 76%. TOF-PDMS (m/z) =507.1 (M^+). HPLC (t_R=5.730 min). ^{1}H-NMR (DMSO-d_6): 1.71—2.38 (m, 11H), 2.74—3.39 (m, 7H), 4.23—4.26 (m, 1H, CH$_{D\text{-}Glu}$), 4.5—4.94 (m, 3H), 7.09—7.50 (m, 8H, aromatic), 7.86+8.08 (d, 1H, J=7.95 Hz, NH$_{D\text{-}Glu}$, *cis*+*trans*: 47/53), 8.32+8.60 (broad, 1H, NH$_{Ipe}$, *cis*+*trans*), 9.83+10.15 (s, 1H, NH$_{paratoluidine}$, *cis*+*trans*), 12.05 (broad, 1H, COOH).

(7) Cyclohexylcarbonyl-D-Tic-D-Glu(NH-paratolyl)-OH: Yield, 75%. TOF-PDMS (m/z)=505.2 (M^+). HPLC (t_R=22.575 min). ^{1}H-NMR (DMSO-d_6): 1.03—2.45 (m, 17H), 2.71—2.84 (m, 1H, CH$_{cyclohexyle}$), 2.99—3.12 (m, 2H, H$_{4\text{-}Tic}$), 4.05—4.10 (m, 1H, CH$_{D\text{-}Glu}$), 4.43+4.63 (d, 1H, J=16.16 Hz, H$_{1\text{-}Tic}$, *cis*+*trans*: 45/55), 4.53+4.72 (d, 1H, J=16.16 Hz, H$_{1\text{-}Tic}$, *cis*+*trans*), 4.74+4.83 (t, 1H, J=5.76 Hz, H$_{3\text{-}Tic}$, *cis*+*trans*), 7.04 (d, 2H, J=8.16 Hz, H$_{paratoluidine}$), 7.08—7.23 (m, 4H, H$_{Tic}$), 7.40 (d, 2H, J=8.16 Hz, H$_{paratoluidine}$), 7.92+8.21 (d, 1H, J=7.74 Hz, NH$_{D\text{-}Glu}$, *cis*+*trans*), 9.62+9.69 (s, 1H, NH$_{paratoluidine}$, *cis*+*trans*), 12.50 (broad, 1H, COOH).

(8) Phenylcarbonyl-D-Tic-D-Glu(NH-paratolyl)-OH: Yield, 83%. TOF-PDMS (m/z)=499.1 (M^+). HPLC (t_R=21.310 min). ^{1}H-NMR (DMSO-d_6): 1.85—2.45 (m, 7H), 3.13—3.25 (m, 2H, H$_{4\text{-}Tic}$), 4.18+4.28 (m, 1H, CH$_{D\text{-}Glu}$, *cis*+*trans*: 45/55), 4.54+4.70 (d, 1H, J=16.30 Hz, H$_{1\text{-}Tic}$, *cis*+*trans*), 4.55+5.04 (m, 1H, H$_{3\text{-}Tic}$, *cis*+*trans*), 4.63+5.05 (d, 1H, J=16.30 Hz, H$_{1\text{-}Tic}$, *cis*+*trans*), 7.01—7.57 (m, 13H, aromatic), 8.41 (t, 1H, J=8.30 Hz, NH$_{D\text{-}Glu}$), 9.70+9.77 (s, 1H, NH$_{paratoluidine}$, *cis*+*trans*).

(9) H-Ipe-L-Tic-D-Glu(NH-paratolyl)-OH : Yield, 70%. TOF-PDMS (m/z) =506.8 (M^+). HPLC (t_R=16.395 min). ^{1}H-NMR (DMSO-d_6): 1.75—2.40 (m, 11H), 2.89—3.37 (m, 7H), 4.09—4.12 (m, 1H, CH$_{D\text{-}Glu}$), 4.42+4.67 (d, 1H, J=16.05 Hz, H$_{1\text{-}Tic}$, *cis*+*trans*: 40/60), 4.90+4.93 (d, 1H, J=16.05 Hz, H$_{1\text{-}Tic}$, *cis*+*trans*), 4.97+5.06 (m, 1H, H$_{3\text{-}Tic}$, *cis*+*trans*), 7.13—7.53 (m, 8H, aromatic), 8.01+8.41 (t, 1H, J=7.48 Hz, NH$_{D\text{-}Glu}$, *cis*+*trans*), 9.84+9.90 (s, 1H, NH$_{paratoluidine}$, *cis*+*trans*).

(10) H-Ipe-D-Tic-L-Glu(NH-paratolyl)-OH: Yield, 65%. TOF-PDMS (m/z) =505.4 (M^+). HPLC (t_R=16.730 min). ^{1}H-NMR (DMSO-d_6): 1.79—2.40 (m, 11H), 2.94—3.35 (m, 7H), 4.10 (m, 1H, CH$_{L\text{-}Glu}$), 4.42+4.67 (d, 1H, J=16.10 Hz, H$_{1\text{-}Tic}$, *cis*+*trans*: 45/55), 4.91+4.94 (d, 1H, J=16.10 Hz, H$_{1\text{-}Tic}$, *cis*+*trans*), 4.99+5.04 (m, 1H, H$_{3\text{-}Tic}$, *cis*+*trans*), 7.12—7.53 (m, 8H, aromatic), 7.88+8.32 (broad, 1H, NH$_{L\text{-}Glu}$, *cis*+*trans*), 9.84+9.90 (s, 1H, NH$_{paratoluidine}$, *cis*+*trans*).

Conformational Analysis The conformational space of H-Ipe-D-Tic-D-Glu(NH-paratolyl)-OH and phenylpropylcarbonyl-L-prolyl-pyrrolidine compounds were explored by systematic torsional driving. Calculations were performed with the DISCOVER module of the MSI software package (Molecular Simulation Inc., San Diego, U.S.A.), and we used the implemented CVFF force field. An initial number of rotamers was associated to each different bond depending on its free rotable or pseudo-rotable character. Two rotamers corresponding to the *cis* and *trans* conformations were associated to the N-terminal amide linkage. For single bonds, a set of three rotamers was considered. For those directly attached to the prolyl or D-Tic ring on the C-terminal side or to the N-terminal amide group, five rotamers were considered. Geometry of proline and D-Tic rings were also altered during the search. With regard to the Ipe amino ring, its initial conformation was taken in a "chair" form. After discarding abnormal conformations, each rotamer was then refined by a molecular mechanics procedure. To take into account solvent effects, solvation energy was calculated according to the BEM method[15] and added to the potential energy term. Redundant conformers were finally eliminated on the basis of energetic and interatomic distances and dihedral angle geometric criteria. Particular interest was focused upon conformers with total energy $E<E_{min}+5$ kcal/mol.

Assay of Tc80 Inhibition Tc80 was purified following two steps of chromatography (DEAE and phenyl-sepharose), as previously reported.[3] Tc80 inhibition was evaluated by using the fluorogenic substrate Suc-Gly–Pro–Leu–Gly–Pro–AMC. A test compound was dissolved in tris–HCl buffer (25 mM, pH=7.5), containing 10% DMSO at various concentrations. The solution (20 μl), was pre-incubated for 15 min with 20 μl of enzyme in the same buffer at 37 °C. The enzymic reaction was then initiated by adding 20 μl of 33 μM substrate solution. After 15 min the reaction was stopped by adding 100 μl of EtOH. The fluorescence of free released AMC was measured at 440 nm upon excitation at 380 nm in a Hitachi 2000 spectrofluorometer. Inhibitory potency was evaluated by the IC_{50} value, which was defined

as the concentration of the test compound that resulted in 50% inhibition of the fluorescence with respect to the DMSO control.

Acknowledgements We are extremely grateful to Dr. Elisabeth Davioud-Charvet for fruitful discussions concerning enzymic and biological studies, Gérard Montagne for NMR experiments and Marie-Isabelle Parent for technical assistance. S.V. is the recipient of a fellowship from the CNRS and Region Nord/Pas-de-Calais.

References

1) World Health Organization (WHO), *Technical Reports Series.*, **811** (1991).
2) Liotta L. A., Rao C. N., Barski S. H., *Lab. Invest.*, **49**, 636—649 (1983).
3) Santana J. M., Grellier P., Schrevel J., Teixeira A., *Biochem. J.*, **325**, 129—137 (1997).
4) Deprez B., Williard X., Bourel L., Coste H., Hyafil F., Tartar A., *J. Am. Chem. Soc.*, **117**, 5405—5406 (1995).
5) Pinilla C., Appel J. R., Blanc P., Houghten R. A., *Biotechniques*, **13**, 901—905 (1992).
6) Dooley C. T., Houghten R. A., *Life Science*, **52**, 1509—1517 (1993).
7) Sano S., Kawanishi S., *J. Am. Chem. Soc.*, **97**, 3480—3484 (1975).
8) Feinberg R. S., Merrifield R. B., *J. Am. Chem. Soc.*, **97**, 3485—3496 (1975).
9) Miranda L. P., Jones A., Meutermans W. D. F., Alewood P. F., *J. Am. Chem. Soc.*, **120**, 1410—1420 (1998).
10) Higuchi N., Saito N., Hashimoto M., Hukami H., Tanaka T., Japan. Patent 201877 (1987) [*Chem. Abstr.*, **108**, 75852m (1988)].
11) Yoshimoto T., Tsuru D., Yamamoto N., Ikesawa R., Furukawa S., *Agric. Biol. Chem.*, **55**, 37—43 (1991).
12) Saito M., Hashimoto M., Kawaguchi N., Shibata H., Fukami H., Tanaka T., Higuchi N., *J. Enzyme. Inhibition*, **5**, 51—75 (1991).
13) Fehér M., Kánai K., Podányi B., Hermecz I., *Quant. Struct.-Act. Relat.*, **16**, 136—141 (1997).
14) Goodman M., Chorev M., *Acc. Chem. Res.*, **12**, 1—7 (1979).
15) Horvath D., Van Belle D., Lippens G., Wodak S. J., *J. Chem. Phys.*, **104**, 6679—6695 (1996).