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AUTOMATED PARALLEL SYNTHESIS OF A TETRAHYDROISOQUINOLIN-BASED LIBRARY : POTENTIAL PROLYL ENDOPEPTIDASE INHIBITORS

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Abstract: Solution-phase automated parallel synthesis of a Tic-based library is described. This library comprising 2560 members, was obtained from the combination of 80 carboxylic acids and 32 amines and was screened against Tc80 protease, a parasitic prolyl endopeptidase secreted by *Trypanosoma cruzi*. Pyrrolidine derivatives proved the most potent inhibitors with IC_{50} values found in the low nanomolar range.

Introduction

The use of parallel synthesis for the production of large arrays of individual compounds is gaining wide acceptance because of the advantages afforded by its application toward accelerated lead optimization. We have recently described the use of orthogonal D-tripeptide combinatorial libraries to discover inhibitors of Tc80 protease, a novel potential target from *Trypanosoma cruzi* involved in host cell invasion.[1-3] The screening of these libraries led to the discovery of a low micromolar inhibitor corresponding to H-Ipe-D-Tic-D-Glu(S-paratolyl)-OH. In addition to substrate specificities, the recognition of Tic residue by Tc80 protease and Tic/Proline analogy suggested that Tc80 protease could be a prolyl endopeptidase (PEP).[3] Moreover, the microsequence analysis of Tc80 was carried out upon several well-defined peptides, resulting from the cleavage of the native Tc80 protein with endoproteinase Lys-C. The most striking sequence similarity (94 % identity), was found with the sequence 630-647 of the catalytic site of human PEP contained in the Swiss Protein Identification Resource Databank.[4] Prolyl endopeptidases (EC 3.4.21.26), also known as prolyl oligopeptidases or post-proline endopeptidases, are serine proteases involved in the cleavage of small biologically active peptides, such as hormones and neuropeptides, but are unable to degrade large proteins.[5] Very recently, the crystal structure of the Z-Pro-prolinal-porcine PEP complex has been elucidated and has been reported to contain an unusual ß-propeller structure involved in the regulation of the catalysis.[6] Substrates are selected by size exclusion at the propeller domain and also by the specificity of the active site, protecting large proteins from proteolysis in the cytosol. In contrast, Tc80 enzyme was shown to exhibit the unusual property of cleaving collagens I and IV in addition to the small peptides commonly used as substrates of PEPs. As a result of the differences in substrate specificities of Tc80 and PEPs, selectivity between parasitic and human PEP towards inhibitors could be expected. In this paper, we report the design and the synthesis of a lead structure containing the Tic moiety, from which we prepared a library using 32 amines and 80 carboxylic acids respectively.

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Identification of a lead structure for optimization

From the hit H-Ipe-D-Tic-D-Glu(S-paratolyl)-OH, were synthesized several analogues, among them the more stable H-Ipe-D-Tic-D-Glu(NH-paratolyl)-OH (compound **1a**, Figure 1).[3] The conformational analysis of compound **1a** and phenylpropylcarbonyl-L-prolyl-pyrrolidine, a potent PEP inhibitor known as SUAM-1221 (compound **1b**),[7-9] permitted the identification of low-energy conformers displaying very high shape similarity.[10] These two molecules can be considered as retro-inverso analogs.[11] The similarities between **1a** and **1b** prompted us first to measure the activity of SUAM-1221 (compound **1b**), on Tc80, then to prepare the L-Tic analogue of SUAM-1221, (phenylpropylcarbonyl-L-Tic-pyrrolidine, compound **1c**). While compound **1c** inhibits Tc80 in the low nanomolar range (IC_{50} = 7 nM), SUAM-1221 was found to display an IC_{50} of 15 nM. Given that the high hydrophobicity of Tic compared to proline can constitute a criterion of specificity between the different PEPs and particularly with the parasitic PEP, we prepared a biased Tic-based library of 2560 compounds in order to study structure-activity relationships.

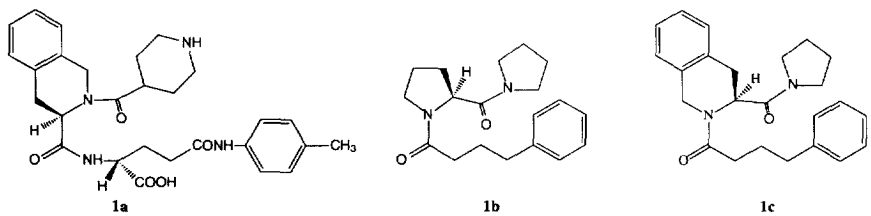


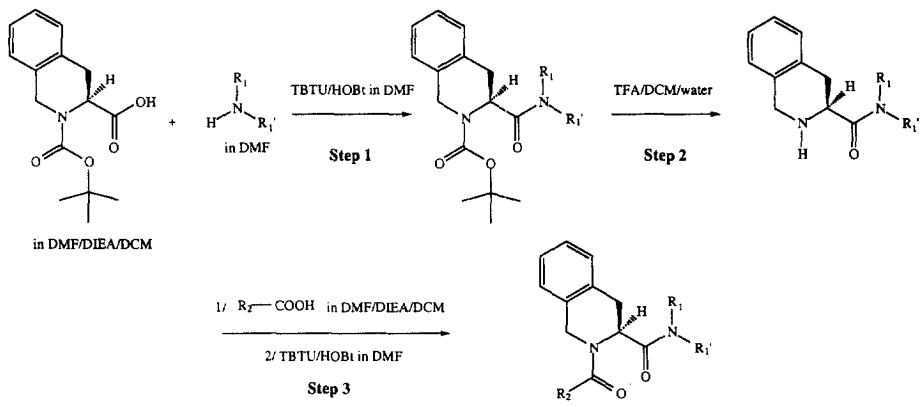
Figure 1. Structure of **1a:** H-Ipe-D-Tic-D-Glu(NH-paratolyl)-OH, **1b:** SUAM-1221, **1c:** phenylpropyl carbonyl-L-Tic-pyrrolidine.

Chemistry and Library Generation

Solution-phase methodology was used for the preparation of compounds according to the general reaction pathway, outlined in Scheme 1. The 32 selected amines dissolved in DMF (0.1 M, 1 eq. DIEA), were automatically coupled in parallel to tert-butyloxycarbonyl-L-Tic dissolved in a DIEA/DCM/DMF mixture (21/8/71), using TBTU and HOBt as coupling reagents, for 4 hours (Step 1). The 32 different arrays obtained, each containing 80 identical compounds, were then evaporated under vacuum. After Boc deprotection with a TFA/DCM/water mixture (50/50/0,5), added manually to each well, then evaporation (Step 2), the 80 acids were automatically coupled, using the same coupling reagents as for the first step (Step 3). A robotic quench, using a mixture of dimethylamine hydrochloride/DIEA/DMF performed for 4 hours and final evaporation, led to 32 arrays each one containing 80 different compounds, yielding a library of 2560 members.

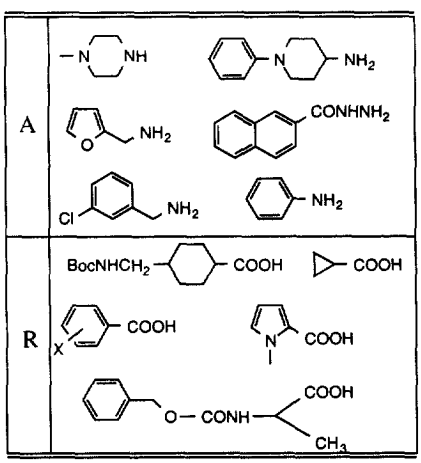
The equipment employed was provided by CEREP, Lille. Reagents were solubilized to the desired concentration using a computer-assisted weighing station, with the aid of a Orca HP robotic arm and a HP diluting system. Reagents were dispensed to the reaction plates using a Tecan Genesis RSP 150. Quality control plates were sampled on the same machine (5 μL per well, 4 wells per plaque plus one entire plaque). Mass spectra and HPLC profiles were obtained on a Micromass Platform LC-MS using a Gilson 215 autosampler and an HP 1050 LC respectively. These data indicated that the desired products were present, along with the identical trifluoroacetic salts, HOBt and TBTU by-products in each well, which proved however, to be inactive towards Tc80; consequently, purity was calculated without including them and was found higher than 85 %.

Scheme 1.

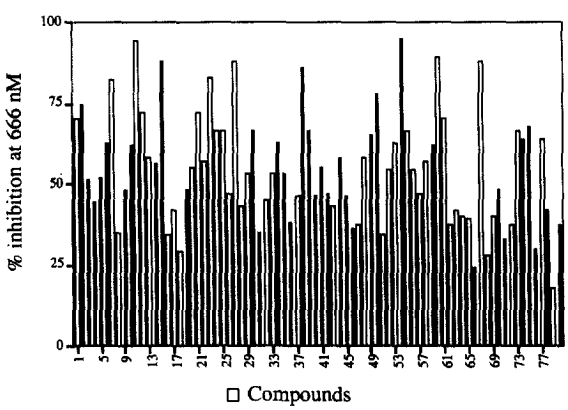


Results and discussion

The library was diluted to a 2 μM concentration in a Tris-HCl buffer containing 10% DMSO and screened at 666 nM against Tc80 prolyl endopeptidase. Pyrrolidine and thiazolidine, both identified as pocket P1-binding moieties in literature studies, are essential in proline derivatives with respect to their conservation of activity. A drastic loss of activity was generally observed when these residues were replaced by bulkier heterocycles, pseudoaromatic heterocycles or open cycles.[12-13] In our library, besides pyrrolidine, 31 amines were chosen for their structural diversity rather than their analogy to pyrrolidine. Thus, several aromatic amines and benzylic amines possessing a variety of substituents on the aromatic ring were used as well as alicyclic amines (N-methyl piperazine, N-aryl piperazine), aromatic or heteroaromatic hydrazides (Scheme 2), and protected amino acids (Leu-O*p*NO_2Bzl, Trp-OBzl, Ala-NH-*p*NO_2phenyl).



Scheme 2. Examples of amines (A) and carboxylic acids (R) displaying respectively no and low inhibitory potency at 666 nM.

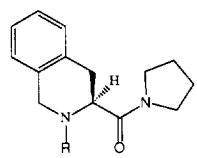


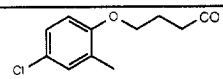
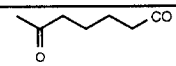
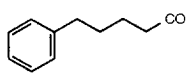
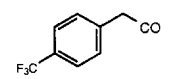
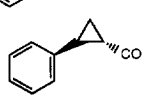
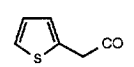
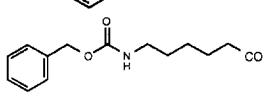
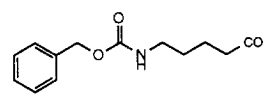
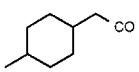
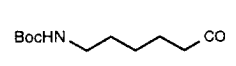
Scheme 3. Screening results for the 80 compounds containing pyrrolidine.

The screening showed that only pyrrolidine derivatives displayed an obvious inhibitory potency which was in good correlation with the results of the literature for the proline series. The Tc80 prolyl endopeptidase appears therefore to possess a similar active site fold as other PEPs concerning its S1 pocket (Scheme 4), although it is capable of cleaving large proteins (collagens).

As shown in Scheme 3, ten compounds among the 80 pyrrolidine derivatives, displayed an inhibition higher than 75% and forty-five an inhibition higher than 50% at 666 nM concentration. The ten most potent inhibitors were synthesized manually, using reagents and coupling agents under the same conditions as for the automatized method. They were purified by preparative TLC and characterized by ^{1}H and ^{13}C NMR, mass spectrometry and HPLC, before being tested. IC_{50} values were evaluated[14] and inhibition percentages were found approximatively in the same range as those found at 666 nM concentration in the preliminary screening (Table 1). This result shows that crude products are pure enough to display an activity quite identical to purified compounds.

Table 1. Inhibitory potency of the ten most potent Tc80 inhibitors from the library, in the presence of 11 μM Suc-Gly-Pro-Leu-Gly-Pro-AMC as substrate.



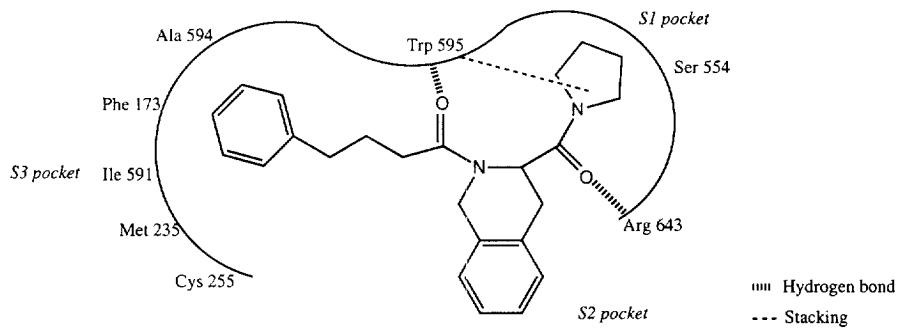
n°	R	% inhibition at 666 nM a	% inhibition at 666 nM b	IC_{50} (nM) b	n°	R	% inhibition at 666 nM a	% inhibition at 666 nM b	IC_{50} (nM) b
1		95	90	17	6		88	87	25
2		94	94	14	7		86	88	21
3		89	86	23	8		83	73	54
4		88	88	21	9		78	85	36
5		88	88	9	10		76	82	55

a: values from primary screening, obtained with crude compounds; b: values obtained with purified compounds. For SUAM-1221, IC_{50} was 15 nM.

Structure-activity relationships were established assuming a tri-dimensional structure of the parasitic enzyme identical to that of the recently described porcine muscle PEP (Scheme 4).[6] The hydrophobic character of the S3 pocket explains the selection of inhibitors with almost all of them, containing a lipophilic arylalkyl side chain at the N-terminal end.[8-9] However, benzoic acid analogues as well as their heterocyclic counterparts (Scheme 2), displayed weak inhibitory potencies which were generally improved by the presence of electron-

withdrawing substituents (nitrile, trifluoromethyl), on the rings. Absence or low activity of benzoic acid analogues can be explained by the steric hindrance of aromatic rings in the formation of the S3P3 hydrogen bond between the carbonyl oxygen of these residues and the side chain of Trp595 (Scheme 4).[6] A drastic decrease of inhibition was also observed with arylalkyl carboxylic acids containing a tertiary α-carbon atom, whether this latter possessed a methyl group, a bulkier Boc or Z-protected amine or is included in a saturated ring. The single exception is compound **3** whose carbonyl group is linked to a cyclopropyl moiety.

The most promising inhibitor was the methylcyclohexane acetic acid derivative **5**, used as a mixture of *cis* and *trans* isomers and displaying an activity (IC_{50} = 9 nM), similar to that of the initial lead **1c**. Isomers were separated and isolated by reverse-phase HPLC.[15] The IC_{50} value of the first isolated isomer was slightly increased (12 nM), whereas the second isomer displayed an improved IC_{50} value (7 nM), compared to the initial mixture. The structural variation between the isomers involves a region less critical for recognition than the central chiral moiety and explains their weak difference of activity. Comparatively, IC_{50} values vary from 7 nM for L-Tic derivative **1c** to 1150 nM for its D-Tic analogue. Stereochemistry of the Tic residue, the putative S2-binding moiety, seems therefore essential for inhibition. This latter feature corroborates the results from the literature: a total decrease of activity is observed when the P2 moiety, belonging to the L series, is replaced by its D-isomer.[16]



Scheme 4. Proposed interactions of **1c** with the active site of Tc80.

In conclusion, this Tic-based library provides new tools of varying hydrophobicity, in the low nanomolar range, to evaluate the role of Tc80 prolyl endopeptidase in the biology of the parasite and particularly in the invasion phase of the host cell. Moreover, the ease of synthesis of these new potent inhibitors makes it possible to obtain readily large quantities for *in vivo* studies.

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10. The conformational spaces of **1a** and **1b** were explored by a systematic torsional driving. Calculations were performed with the DISCOVER module of the MSI software package and 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 of 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 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.

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14. PEP 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 minutes 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 minutes 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 microplate fluorometer. 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.

15. Preparative RP-HPLC was performed on a Beckman chromatograph using a 20x1 cm Zorbac column packed with C3 silica. Separation was achieved with a gradient of (A) and (B): 0-30 % (B) during 1 minute, then 30-80 % (B) in 50 minutes, at a flow rate of 3 mL/min, with UV detection at 220 nm. The following solvent system was used: (A): 0.05 % trifluoroacetic acid (TFA) in H_2O; solvent (B): 0.05 % TFA, 20 % H_2O, 80% acetonitrile.

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