Synthesis, screening and computational investigation of pentacycloundecane-peptoids as potent CSA-HIV PR inhibitors

ARTICLE *in* EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY · JULY 2012

Impact Factor: 3.45 · DOI: 10.1016/j.ejmech.2012.06.019 · Source: PubMed

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European Journal of Medicinal Chemistry xxx (2011) 1–10

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Original article

Synthesis and structural studies of pentacycloundecane-based HIV-1 PR inhibitors: A hybrid 2D NMR and docking/QM/MM/MD approach

Maya M. Makatini [a], Katja Petzold [b], Shimoga N. Sriharsha [b], N. Ndlovu [b], Mahmoud E.S. Soliman [c,**], Bahareh Honarparvar [b], Raveen Parboosing [a,d], Anneta Naidoo [d], Per I. Arvidsson [a,e,f], Yasien Sayed [g], Patrick Govender [h], Glenn E.M. Maguire [b], Hendrik G. Kruger [b,*], Thavendran Govender [a,***]

[a] School of Pharmacy and Pharmacology, University of KwaZulu-Natal, Durban 4001, South Africa
[b] School of Chemistry, University of KwaZulu-Natal, Varsity Drive, Durban 4001, South Africa
[c] Department of Pharmaceutical Organic Chemistry, Faculty of Pharmacy, Zagazig University, Zagazig 44519, Egypt
[d] Department of Virology, National Health Laboratory Service, University of KwaZulu-Natal, Durban, South Africa
[e] Organic Pharmaceutical Chemistry, Department of Medicinal Chemistry, Uppsala Biomedical Centre, Uppsala University, Box 574, SE-751 23 Uppsala, Sweden
[f] Discovery CNS and Pain Control, AstraZeneca R&D Sodertalje, S-151 85 Sodertalje, Sweden
[g] Protein Structure-Function Research Unit, School of Molecular and Cell Biology, University of the Witwatersrand, Johannesburg 2050, South Africa
[h] School of Biochemistry, Genetics and Microbiology, University of KwaZulu-Natal, Durban 4001, South Africa

ARTICLE INFO

Article history:
Received 17 February 2011
Received in revised form
26 May 2011
Accepted 28 May 2011
Available online xxx

Keywords:
Pentacycloundecane (PCU) peptides
HIV protease inhibitor
EASY–ROESY
Docking
QM/MM/MD
Molecular dynamics simulation

ABSTRACT

Pentacycloundecane (PCU) lactam-peptide based HIV protease inhibitors were synthesized and nanomolar activity against the resistance-prone wild type C-South African HIV protease is reported. NMR investigations indicated that the activity is related to the chirality of the PCU moiety and its ability to induce conformations of the coupled peptide side chain. EASY-ROESY NMR experiments gave information about the 3D structure of the cage peptides and 3D solution structure could be linked to the experimental IC_{50} activity profile of the considered inhibitors. QM/MM/MD simulations of the inhibitors in solution confirmed the NMR observed conformations. Docking experiments and QM/MM/MD simulations of the inhibitor-HIV PR complexes were also performed. These computational results complimented the experimental inhibition activities and enabled us to report a unique binding mode for PCU-based inhibitors at the active site of HIV-protease enzyme. A conserved hydrogen bonding pattern between the norstatine type functional group of the PCU hydroxylactam and active site residues, ASP25/ASP25′, was observed in all active compounds. The biological significance and possible mode of inhibition by PCU-based HIV PR inhibitors discussed herein provide us with a deeper understanding of the mode of action of these novel inhibitors. The PCU-peptides are between 6000 and 8500 time less toxic to human MT-4 cells than Lopinavir. This potentially creates new application avenues for these putative inhibitors to be investigated against a vast number of other disease-related proteases.

1. Introduction

In the last few years, experimental findings indicate the rapid emergence of drug resistance to most of the HIV-1 PR inhibitors, because site specific mutations in the enzyme occur at one or more positions [1,2]. This drug resistance has led to an urgent demand for new drug candidates [1,2]. As a result numerous potent human immunodeficiency virus type 1 aspartic protease (HIV-1 PR) inhibitors have been approved as drugs for the inhibition of this enzyme by the Food and Drug Administration, while several others are under clinical investigation [3,4]. Since most of these drugs are peptidic in nature, their oral bioavailability and half-life are limited [5].

The HIV-protease enzyme is a C_2-symmetric active homo-dimer responsible for the cleavage of *gag* and *gag-pol* polyproteins into their functional constituent proteins. The active site of HIV PR is composed of catalytic aspartic acid residues Asp25 and Asp25′ [6]. The HIV protease subtype C is predominantly found in South Africa (C-SA) and shows rapid development of resistance to common drugs. The design and testing of most HIV-PR inhibitors were optimized on the subtype B strain, prevalent in Europe and Northern America [1].

Abbreviations: PIs, protease inhibitors; HIV PR, HIV protease; AZT, azidothymidine; CNS, central nervous system; PCU, pentacycloundecane.

* Corresponding author.
** Corresponding author.
*** Corresponding author.
E-mail addresses: mess20@bath.ac.uk (M.E.S. Soliman), kruger@ukzn.ac.za (H.G. Kruger), govenderthav@ukzn.ac.za (T. Govender).

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doi:10.1016/j.ejmech.2011.05.071

The incorporation of polycyclic bulky hydrophobic cage frameworks into biologically active molecules was reported to enhance their activity [7–11] and retard biodegradation thereof [12]. Various cage systems have shown biological activity [8,9,13,14]. It also enhances the transport of drugs attached to it across cell membranes [8,15,16]. Adamantane was previously coupled to azidothymidine (AZT) and displayed significant enhancement of drug concentration in the central nervous system (CNS) [8,17]. Brookes et al. [12] suggested that the steric bulk of the cage should increase the regiospecificity [18–20] of the drug to particular receptor sites. This was confirmed by Geldenhuys et al. several years later [8]. This finding was also confirmed by our recent study of various adamantane diamine compounds designed as potential anti-tuberculosis drugs [21].

Kempf et al. reported a 20 fold increase in binding affinity to the recombinant HIV-PR [22] catalytic site of the (*R*,*R*)- and (*R*,*S*)-dihydroxyethylene transition-state isostere compared to the (*S*,*S*)-conformation [23]. This implies that the stereochemistry of molecules plays an important role in modulating their biological activity for specific binding sites. The importance of chiral influences has been reported extensively in drug design [24–26], for example, Thalidomide [27]. Interestingly, until now there is no compelling evidence that enantiomeric cage compounds give any difference in selectivity toward receptor sites [28,29].

From a structural point of view, the active site of the HIV-1 PR is approximately an open-ended cylindrical hydrophobic cavity of about 10 Å diameter [6] and the S1 and S1′ sub-binding site of the HIV-PR can accommodate bulky hydrophobic substituents [30]. With this in mind, it was predicted that the non-conventional bulky PCU-lactam structure [31–35] could potentially occupy these sub-sites. Even though the S2 and S2′ pockets are of a hydrophobic nature, both hydrophilic and hydrophobic side chains from the *gag* and *gag-pol* polyproteins can occupy these sites [36]. Based on literature and the HIV substrate sequence, it was also envisaged that either the glutamic acid or alanine residues from the inhibitor peptide chain, would be a potential anchor to the S2 and S2′ sub-sites.

Bearing all these concepts in mind, we have reported novel PCU lactam-peptide derivatives as potential transition-state analogues for HIV-PR inhibition [37]. The PCU-lactam contains a hydroxyl carbonyl functional group, resembling a norstatine transition-state isostere (Fig. 1) [30]. Furthermore, it consists of a very stable amide bond [33] situated at the axial position of a rigid cyclohexane boat structure (Fig. 3: C-1–C-7–C-9–C-8–C-10–C-11) which can potentially interact with the catalytic residues in the active site. We coupled various peptide sequences to the racemic PCU-cage. The first sequence was selected from a natural HIV-protease substrate, FEAIS [38] where F is replaced with the cage lactam, to potentially ensure high specificity of the proposed inhibitor to the active site. This peptide sequence was then systematically varied.

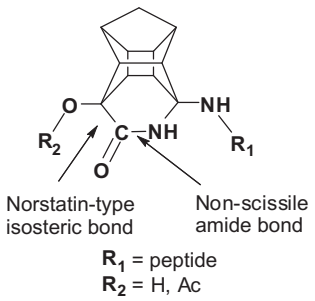

Fig. 1. Pentacycloundecane scaffold. Representing the PCU lactam-peptide derivatives as novel potential transition-state analogues for HIV-PR inhibition.

Herein, we present the full report of the synthesis, biological activity, NMR and computational investigation of seven novel PCU derived HIV protease inhibitors.

2. Material and methods

2.1. Experimental details

2.1.1. PCU-peptide synthesis

Peptides were synthesized on a CEM automated microwave assisted peptide synthesizer on 2-chlorotrityl-chloride resin *via* the Fmoc strategy. The desired crude peptides, all (*S*)-amino acids, were cleaved from the resin and coupled to the PCU lactam in solution. Purification of the peptides was achieved *via* semi-preparative HPLC. Only one of the diastereomeric cage peptide mixtures could be separated. The overall yields for the cage peptides were between 17 and 47%. Detailed yields, methods for the synthesis and purification are described in the Supplementary material.

2.1.2. Optical rotation (OR) and circular dichroism (CD)

Measurements were recorded on a Perkin–Elmer Polarimeter (Model 341) and Jasco J-810 CD spectrometer instruments, respectively, and all compounds were dissolved in MeOH. Optical rotation was measured at 589 nm and the CD spectra were recorded from 190 to 260 nm.

2.1.3. NMR

Samples were dissolved in 450 μl of deuterated DMSO-d6 and measured at 25 °C. All ^{1}H, ^{13}C, COSY, HMBC and HSQC NMR spectra, required for assignment were recorded on a Bruker AVANCE III 400 MHz spectrometer, BBO probe with z-gradient with standard parameters. The EASY–ROESY [39] measurements required for structural analysis were recorded on a Bruker AVANCE III 600 MHz spectrometer using a BBO probe with z-gradient at a transmitter frequency of 600.1 MHz (spectral width, 10.0143 Hz; acquisition time, 0.1704436 s; 90° pulse width, 11.02 μs; scans, 8; relaxation delay, 2.0 s, mixing time 0.12 s, 2048 points in *F*2 dimensions). Processing and assignments were carried out using the Topspin 2.3 software from Bruker Karlsruhe. In order to determine if the same 3D structural conformation observed for the inhibitors in DMSO are also valid for the HIV-PR activity studies, the NMR spectra for PCU-EAIS were recorded in a buffered D_2O solution. All spectra mentioned in the text are available with the Supplementary material.

2.1.4. In vitro HIV-1 protease inhibition assay

Synthesis and purification of the HIV protease type C were performed as described in the Supplementary material [4,40,41]. The catalytic activity of the protease was monitored following the hydrolysis of chromogenic peptide substrate, Ala-Arg-Val-Nle-*p*-nitro-Phe-Glu-Ala-Nle-NH_2 at 300 nm using an Analytik Jena Specord 210 spectrophotometer. Activity was standardized using commercially available drugs Atazanavir and Lopinavir. All cage peptides were soluble in the aqueous buffer solution (pH = 5, 50 mM sodium acetate and 0.1 M NaCl).

2.1.5. XTT screening assay

The *in vitro* toxicology XTT (2,3-bis[2-Methoxy-4-nitro-5-sulfophenyl]-2*H*-tetrazolium-5-carboxyanilide) Kit (Sigma–Aldrich, Missouri, USA) was used to determine the cytotoxicity of the compounds, according to well described methods [42,43]. The XTT assay is based on the activity of mitochondrial enzymes of viable cells which cleave the tetrazolium ring of XTT yielding orange formazan crystals. Formazan crystals are soluble in aqueous solutions and the orange color can be spectrophotometrically measured. A decrease in cell viability results in a proportional reduction in the amount of

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formazan formed, indicating the degree of cytotoxicity caused by the compound.

The assay was performed as follows: MT-4 cells were cultured in RPMI-1640, 10% fetal calf serum, 1% penicillin, 1% streptomycin and 1% Fungizone® (Aphotericin-B) at 37 °C and 5% CO_2. MT-4 cells were then seeded into the wells of 96-well culture plates at 6×10^5 cells/ml. The cells were then exposed to the compound and eight 5-fold dilutions of the compound. The plate was incubated for 5 days at 37 °C in a CO_2 incubator (5% CO_2).

After 5 days, the XTT salt was added to the wells and incubated for 4 h at 37 °C in a CO_2 incubator. Formazan production was quantified by measuring absorbance at 450 nm (reference wavelength 620 nm). Cytotoxicity was determined by calculating the Inhibitory Concentration at 50% (IC_{50}). The assay was performed in triplicate and Lopinavir was used as a reference drug.

MT-4 cells were obtained from Dr. Douglas Richman at the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: H9/HTLV-IIIB NIH 1983.

2.2. Molecular modeling and computations

2.2.1. QM/MM/MD simulations of CPU-peptide inhibitors in water

The structures of the selected cage diastereomeric compounds, PCU-AISa/b and PCU-EAISa/b were constructed using the Avogadro software [44]. A β-sheet conformation was constructed as the starting structure for the conformational studies.

To determine the low energy conformations of the inhibitors in aqueous solution, the structures of the studied PCU-peptide inhibitors were subjected to hybrid quantum mechanical, molecular mechanics and molecular dynamics (QM/MM/MD) simulations [45]. Gas phase AM1 optimized structures of the inhibitors were enveloped in a cubic box of TIP3P water [46] of side-length 31.4 Å. The energies of the considered inhibitors in solution were minimized to a residual gradient of less than 0.001 kJ mol^{-1} $Å^{-1}$ with a QM/MM method using the DYNAMO package [47]. The QM region (PCU-AIS, 70 atoms and PCU-EAIS, 86 atoms) was described by AM1 semi-empirical Hamiltonian [48]. The entire system was then equilibrated without any constraints by MD for 50 ps at 300 K, using the NVT ensemble prior to 1 ns of separate production MD runs at 300 K, 400 K, 500 K and 600 K (1 fs time step, switched cut off radius of 16 Å applied to all interactions).

2.2.2. C-SA PR enzyme model system

Since the X-ray structure of the South Africa HIV protease subtype C (C-SA) has not yet been reported, the initial 3D structure of the enzyme was taken from the reported X-ray data of subtype B HIV-PR (PDB accession code 1HXW) [49]. C-SA HIV-PR differs from subtype B at eight positions; T12S, I15V, L19I, M36I, R41K, H69K, L89M and I93L [50]. The X-ray crystallographic coordinates of 1HXW were modified as follows:

The original substrate included in the crystal structure, Ritonavir, and the crystallographic water molecules were removed and hydrogen atoms were added to the system. Ionization states for ionizable amino acid residues were determined according to their standard pK_a values. Mutations at eight positions, T12S, I15V, L19I, M36I, R41K, H69K, L89M and I93L were manually induced. Throughout this work, the modeled C-SA HIV-PR was used for docking studies and QM/MM/MD simulations.

2.2.3. Docking of the inhibitors into the C-SA PR model

The 10 lowest energy conformers from the QM/MM/MD simulations of inhibitors in water were used in docking simulations and only the docked complex with the lowest binding energy was considered for further MD simulations. The effect of ionization states of docked compounds to the binding scores has been discussed in literature [51–53]. The ionization states of cage peptide inhibitors have not yet been determined experimentally. During docking, the neutral and ionized states of the inhibitors (aliphatic amine and carboxylic acid groups of compounds to be docked were protonated and deprotonated, respectively) were separately considered and compared. The use of neutral and ionized molecules did not significantly affect the docking results, thus the neutral state of the inhibitors was used for further investigations.

The simulations were performed under physiological pH conditions, which required ensuring the correct protonation state of ionizable groups in the enzyme. In particular, one of the aspartates (Asp25) of the catalytic site exhibits an increased pK_a value of 5.2 in the inhibitor-bound protease [54] while no increased pK_a was reported for the free form of the protease (pK_a = 4.5) [55]. Therefore, a fully deprotonated active site should be prevalent at a physiological pH, and thus this protonation state was used throughout this study. However, to ensure that the results of the simulations do not critically depend on the protonation state, a control simulation of a cage-bound CSA-HIV-1 protease with a protonated carboxylate oxygen of one of the Asp25 residues was also performed.

Docking studies were performed using the Autodock software [56]. Geisteger charges were computed and the Autodock atom types were defined using Autodock Tools, graphical user interface of Autodock supplied by MGL Tools [57]. The Lamarckian Genetic Algorithm (LGA), which is considered as one of the best docking methods available in Autodock [56,58] was employed. This algorithm yields superior docking performance compared to simulated annealing or the simple genetic algorithm and the other search algorithms available in Autodock4. The docked conformations of each ligand were ranked into clusters based on the binding energy. The top ranked conformations was visually analyzed. The suitability of the constructed C-SA PR for docking experiments was tested with a control experiment in which Ritonavir, was docked into active site of the modeled enzyme and compared to the X-ray crystal structure [49] of the Ritonavir–protease complex.

2.2.4. QM/MM/MD simulation of the CPU-peptide enzyme complexes

The docked inhibitor-enzyme complexes with the lowest binding energy were then subjected to QM/MM/MD simulations. The QM region comprised of the inhibitors and the two catalytic residues, ASP25 and ASP25′, was described by the AM1 semi-empirical Hamiltonian. The MM region contained the rest of the enzyme and was described by the OPLSAA potential [59]. QM link atoms were placed along the Cβ(QM)–Cα(MM) bonds of Asp25 and Asp25′. The QM/MM/MD simulations consisted of the following steps: A gas-phase QM/MM MD simulation (NVT, 300 K, 5 ps) was performed to pre-equilibrate the protein and to allow the the binding cavity to accommodate the substrate. The whole system was enveloped in a cubic box of TIP3P water [46] of side-length 79.5 Å with periodic boundary conditions [60]. First, all water molecules were relaxed with a gradient minimizer, while keeping the protein structure frozen. The system was then subjected to a short MD pre-equilibration using mild constraining forces to maintain the desired interactions between the substrate and the catalytic residues. Next, the whole system was equilibrated using MD for 20 ps at 150 K, using the NVT ensemble, still with the protein frozen. Then the entire system was freely minimized without any constraints and subsequently equilibrated for 20 ps at 300 K. Finally, a no-constraint production MD run for 300 ps at 300 K was performed, where hydrogen bond interaction analysis and inhibitor-enzyme electrostatic interactions were monitored

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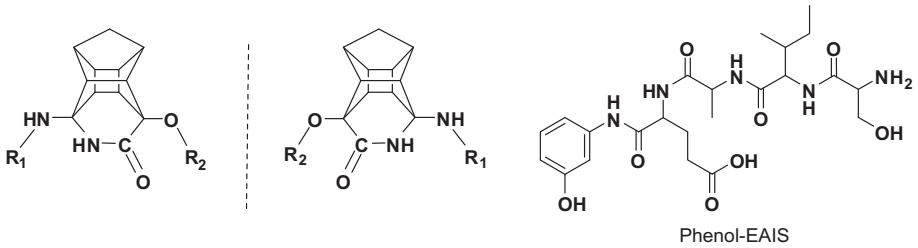

Fig. 2. Diastereomers of PCU. Shown here are the diastereomers of the PCU-derivatives with the substitutions of R_1 and R_2, which properties are presented in Table 1, as well as the control peptide phenol-EAIS.

along the MD trajectories. All structures mentioned in the text are available in PDB format with the Supplementary material.

3. Results and discussion

3.1. Chemistry and biological testing

The PCU-peptides were obtained as diastereomers from the coupling of the enantiopure short peptide to the racemic PCU-lactam (Fig. 2). The sequences of the different inhibitor PCU-peptides are specified in Table 1. All inhibitors were characterized with high-resolution mass spectrometry and NMR. The peptides were tested for HIV-PR inhibition activity (Table 1).

Interestingly, the first cage peptide sequence (PCU-EAIS) that was chosen from the natural HIV-protease substrate (FEAIS [38] where F is replaced with the cage lactam) displayed the best inhibition activity ($IC_{50} = 78$ nM). To confirm the significance of the role of the cage in the inhibition of the HIV-PR active center it was replaced with hydroxyl-phenylamine to produce the control peptide (Phenol-EAIS), which had an $IC_{50} = 10.0$ μM, a 120 fold decrease when compared to PCU-EAIS. Furthermore, the hydroxyl group at position C8 of the PCU-cage is essential, because when we acetylated this functional group, the activity was completely lost (Ac-PCU-EAIS with $IC_{50} > 10.0$ μM).

Systematic removal or substitution of the peptide amino acid residues resulted in a decrease in inhibitory activity. Removal of the serine segment resulted in a six fold decrease (PCU-EAI with $IC_{50} = 0.5$ μM), the substitution of alanine with valine to PCU-EVIS caused a 25 fold decrease. Substitution of the glutamic acid in PCU-EAIS with glutamine gave PCU-QAIS with a 64 fold decrease in activity. Removal of the glutamic acid yielded diastereoisomers PCU-AISa ($IC_{50} = 0.5$ μM) and PCU-AISb ($IC_{50} = 10.0$ μM), which were separated by preparative HPLC. These diastereomers exhibited a 20-fold difference in activity with a 6 fold and 120 fold reduction in respective activities, compared to PCU-EAIS.

Table 1
IC_{50} values for the inhibition of wild type C-SA HIV-1 protease by PCU-lactam peptides and control compounds (Fig. 2). Structural information from EASY–ROESY NMR and binding energies from docking experiments are also presented.

PCU-R_1	R_2	Yield/%	Cage–peptide interaction (CP)[d]	Peptide–peptide interaction (PP)[e]	IC_{50}/μM
PCU-EAIS[a]	H	47	Yes	–	0.078 ± 0.0035
Phenol-EAIS[b]		87	–	–	10.0 ± 3.53
PCU-EAIS[a]	Ac	27	–	–	>10
PCU-EAI[a]	H	23	Yes	–	0.5 ± 0.035
PCU-EVIS[a]	H	33	Yes	–	2.0 ± 0.18
PCU-QAIS[a]	H	20	–	–	5.0 ± 0.71
PCU-AISa[c]	H	18	Yes	–	0.5 ± 0.035
PCU-AISb[c]	H	17	–	Yes	10.0 ± 1.06
Atazanavir					0.004 ± 0.00071
Lopinavir					0.025 ± 0.0014

[a] Diastereomeric mixtures.
[b] Enantiopure.
[c] Diastereomer was separated with semi-preparative HPLC.
[d] CP: ROE signal of the Cage protons with a Peptide NH in solution was observed.
[e] PP: ROE signal of the same Peptide NH with the Peptide side chain in solution was observed.

The cytotoxicity results revealed that the PCU-peptides are between 6000 and 8500 time less toxic to human MT-4 cells than Lopinavir (PCU-EAIS = 218 μm; PCU-EAI = 216 μm; PCU-AISa >300 μm and Lopinavir = 0.035 μm).

These results clearly confirm the significant bioactive contribution of the cage, with a non cleavable norstatine-type bond and the importance of the appropriate peptide sequence for enhanced and specific binding to the active site.

NMR and computational chemistry techniques were employed to determine a potential correlation between the IC_{50} results and the 3D structure of the corresponding inhibitors in solution. ^1H and ^{13}C spectra, 2D ^1H, ^1H correlation and ^1H, ^{13}C correlation spectra were acquired to validate the chemical structure of all compounds (NMR assignments are presented within the Supplementary material).

The NMR elucidation of the mixture of PCU-EAIS diastereomers was challenging. First the diastereomers could not be separated and second it was clear from the data that each of the two diastereomers also exhibits different conformational preferences. It therefore made sense to first assign the signals for the separated diastereomers PCU-AISa and b. In addition, these diastereomers exhibited a 20-fold difference in IC_{50}, which would enable us to investigate the influence of the cage-chirality on HIV PR inhibition by means of applied high-resolution heteronuclear NMR techniques. The ^1H and ^{13}C NMR spectra of PCU-AISa and PCU-AISb were very similar except for carbons C-1 and C-10 of the PCU-cage (Fig. 3).

The ^1H, ^{13}C-HSQC spectrum of PCU-AISa showed that carbons C-1 and C-10 of the cage are split, which was not observed in the spectrum of PCU-AISb. Upon heating, the splitting was reduced and disappeared completely at 333 K indicating that different inter-convertible conformations exist. The EASY–ROESY [39] spectrum enabled us to detect ^1H, ^1H through space ROE interactions of the various inhibitors. PCU-AISa showed long-range correlations between the NHc amide protons of alanine with the PCU protons H-1, H-10 and H-3 and the side chain methyl protons of alanine (H-3′) also showed a correlation with H-3 of the cage (Fig. 3). This dominant conformation was named CP (long-range cage peptide interaction). Therefore the splitting of the cage carbon signals C-10 and C-1 of PCU-AISa was attributed to the existence of at least two conformations of the peptide side chain. In the dominant conformation (CP, about 65%), a through space shielding of C-1 and C-10 exists as a result of the interaction between one of the split alanine amide proton signals (NHc, 8.03 ppm) and the cage protons H-1 and H-10. We observed a minor peptide conformation (about 35%) for PCU-AISa, in which one more alanine-NHc signals (8.07 ppm) is registered. These conformations are most certainly induced by the chiral cage structure and are remarkably stable in

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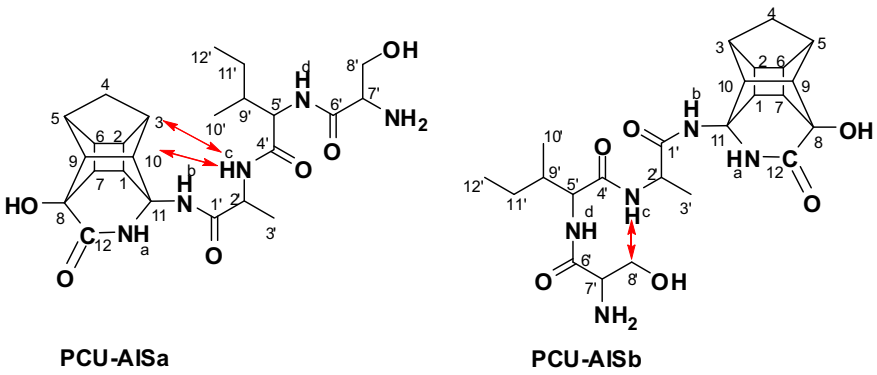

Fig. 3. Long-range interactions. Dominant through-space long-range correlations (indicated by red arrows) observed applying the EASY–ROESY [39] experiment for the two cage-peptide diastereomers PCU-AISa and b in solution. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

view of the high temperature (333 K) required to obtain interconversion (natural short peptides rotates freely at room temperature).

PCU-AISb showed only one set of C-1 and C-10 resonances in the ^{1}H, ^{13}C-HSQC, coinciding with the minor conformation of PCU-AISa. It was clear from the EASY–ROESY spectra that CP interactions for PCU-AISb were absent. Interestingly, a dominant ROE interaction of the alanine-NHc (8.10 ppm) with the serine side chain (H-8′ – Fig. 3) was observed for PCU-AISb; which was not observed for PCU-AISa. This long-range peptide–peptide interaction was named PP. We therefore concluded that the chirality of the cage induces different dominant secondary structures of the peptide chains, either interacting with the cage (CP) or with the peptide itself (PP).

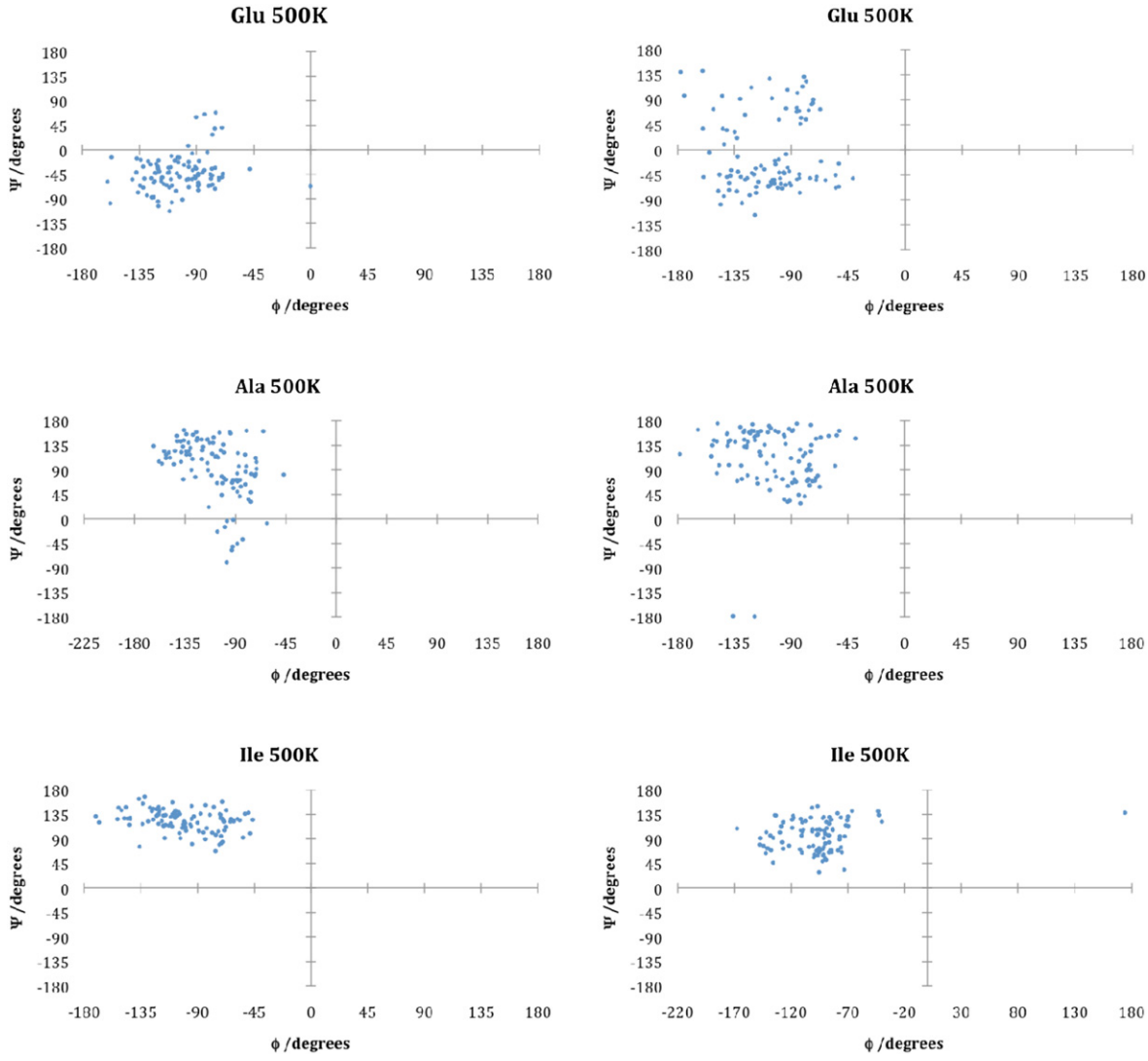

Fig. 4. Ramachandran plots for the three amino acids in PCU-EAISa (left) and PCU-EAISb (right) at 500 K. Data from the 100 lowest energy structures was plotted.

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The difference in HIV-PR inhibition activities of these diastereomers (PCU-AISa is 20 times more active that PCU-AISb) seems to suggest that the peptide conformations induced by the cage enantiomers can be used as a crude tool to predict HIV-PR activity of this family of inhibitors. It is important to note that the dominant low energy conformation of a peptide in solution is not automatically the same conformation of the inhibitor inside the active enzymatic pocket.

Applying the knowledge obtained from elucidating the NMR spectra of PCU-AIS enabled us to solve the intriguing NMR spectra of the PCU-EAIS mixture. The EASY–ROESY spectrum of the most potent inhibitor, the diastereomeric mixture of PCU-EAIS, displayed the same dominant CP conformation (about 79%) present for PCU-AISa. We could not detect the PP conformation as present in PCU-AISb, but a minor conformation (about 21%), most likely and elongated side chain is present. This can also explain the splitting of C-1 and C-10. To prove that the CP conformation is biological relevant and present in the conditions of binding to the HIV-PR, NMR spectra of PCU-EAIS were also recorded in an aqueous buffer (D_2O) used for the HIV-PR inhibition assay; similar EASY–ROESY correlations and HSQC spectra were observed as with DMSO (Table 11 in the Supplementary material).

The ROESY spectra of compounds PCU-EAI and PCU-EVIS also only revealed the CP conformation, but not the PP conformation. The ROESY spectra of PCU-QAIS and PCU-AISb showed no sign of CP interactions and these peptides were found to be the weakest inhibitors in the group. This chiral induced conformation of the cage with the peptide chain is crucial for better recognition and/or binding.

3.2. Computational simulations

To fully explore the molecular and biological behavior of the synthesized CPU-peptide inhibitors, we embarked on a computational chemistry investigation involving three different approaches. First, hybrid QM/MM/MD simulations were used to determine the predominant low energy conformations for selected cage peptide systems in aqueous solution. Second, the PCU-peptides were subjected to docking studies, and finally QM/MM/MD simulations of the docked inhibitor-enzyme complexes were performed.

3.2.1. QM/MM/MD simulations in water

We have previously reported that cage peptides required much higher MD simulation temperatures than room temperature (298 K) for a reasonable exploration of the conformational profile [61,62]. Our NMR results indicated that the two different conformations for PCU-AISa required heating to 333 K to overcome the energy barrier between them. The MD simulations for all cage peptides (PCU-EAISa/b and PCU-AISa/b) were therefore executed at 300 K, 400 K, 500 K and 600 K. The QM/MM/MD results showed that the whole conformational space of each peptide was only fully explored at 500 K. The Ramachandran plots and the 10 lowest energy structures for each of the diastereomeric peptides were analyzed (Fig. 4 and Supplementary material). The Ramachandran plots at 500 K for PCU-EAISa revealed that only the first amino segment (Glu) favored an α-helical character (CP interaction is possible) while the other segments (Ala and Ile) both exhibited β-sheet conformations (CP interaction is not possible). The Glu segment of PCU-EAISb showed both α-helical β-sheet and character at 500 K while at 600 K largely β-sheet character was observed.

Analysis of the l0 lowest conformations of each of the cage peptides from the MD simulation (500K) also confirmed the NMR observations. Nine out of the ten lowest energy structures from PCU-EAISa demonstrated cage peptide (CP) interactions (distances less than 5.0 Å) while only three of lowest energy structures of PCU-EAISb showed this conformation.

Table 2
Selected automated docking results for the synthesized PCU inhibitors and comparison to the experimental HIV-PR inhibition results. (The docked inhibitor-enzyme complexes results are available in PDB format and is provided with the Supplementary material).

Compound	Binding energy[a] (kcal/mol)	IC_{50}/μM
PCU-EAISa	−10.23	0.078 ± 0.0035[b]
PCU-EAISb	−9.81	0.078 ± 0.0035[b]
PCU-AISa	−9.41	0.5 ± 0.035
PCU-AISb	−9.27	10.0 ± 1.06
Ac-PCU-EAISa	−7.06	>10
Ac-PCU-AISa	−6.59	–

[a] Energy calculated from Autodock.
[b] These two diastereomers were not separated and the IC_{50} values are for the mixture.

Similar observations were made for PCU-AISa and b. The alanine segment of PCU-AISa adopted a α-helical conformation at 500 K, while that of PCU-AISb exhibited a β-sheet conformation. The same trend holds for the isoleucine segment.

These MD results therefore provide further evidence for the existence of the conformations observed with the EASY–ROESY experiments. It also suggests that the most active inhibitor (PCU-EAISa) is most likely twice as active as the mixture of diastereomers, since PCU-EAISb does not exhibit the same CP conformation as all the active inhibitors and is therefore most likely much less active.

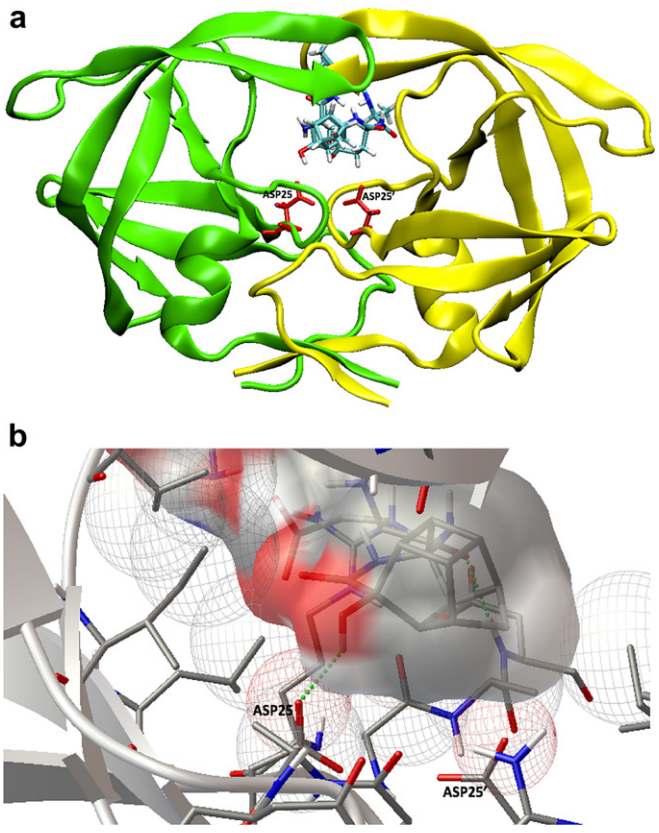

Fig. 5. PCU-EAISa docked to HIV protease. (a) Lowest energy docked structure for PCU-EAISa (CP conformation) with the C-SA HIV-PR. (b) A closer view showing the binding mode of PCU-EAISa inside C-SA HIV-PR active site. The two PR monomers are colored in yellow and green ribbon, the inhibitor presented as colored sticks and the two Asp25 residues are presented as red sticks. The 3D presentations for computational results are available as PDB files with the Supplementary material. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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3.2.2. Docking results

Docking studies are used at different stages in drug discovery such as in the prediction of ligand–receptor complex structures and also to rank the ligand molecules based upon the binding energies of the corresponding ligand–enzyme complexes. Docking protocols aid in elucidation of the most energetically favorable binding mode of the ligand to the receptor. The objective of our docking study was to elucidate the potential interaction mode of the PCU-peptide derivatives with C-SA HIV PR.

The binding energies of the docked PCU-peptide inhibitors have been tabulated (Table 2). Even though Autodock employs the Lamarckian genetic algorithm which is validated for small molecules we wanted to confirm that our docking method works with a well established system and also that the newly generated C-SA enzyme functions well with a known inhibitor. Docking of Ritonavir (cocrystallized inhibitor) with subtype B HIV-PR (PDB accession code 1HXW) [49] was performed to evaluate the efficacy of Autodock for its use in docking experiments with this subtype as a target.

Next, a control docking experiment of Ritonavir with C-SA PR was performed. The docked complex orientation of Ritonavar gave a reasonable RMSD of 0.559 Å with the orientation of Ritonavir from the X-ray structure [49] with HIV-PR type B. The interacting amino acids in C-SA starting structure were conserved in the conformation of the enzyme after docking and the variation in orientation of these amino acids in the binding cavity was unnoticeable.

Docking routines of the new PCU-peptide inhibitors with the C-SA enzyme were then performed. For the most potent inhibitor PCU-EAISa, the hydroxyl group of the PCU-lactam (the proposed norstatine moiety) formed a hydrogen bond with Asp25 of the dimeric catalytic triad residues Asp25-Thr26-Gly27 (A/B chains), most noticeable Asp25 and Asp25′ (Figs. 5 and 6 and the Supplementary material). PCU-EAISa and b also exhibited the lowest binding energies, which correlates with the experimentally observed activity results.

The docked structures of Ac-PCU-EAISa and Ac-PCU-EAISb failed to attain any interaction between the cage lactam and the two Asp25 segments of the PR, which reveals the critical importance of the hydroxyl group of the PCU-lactam for effective binding with the PR.

PCU-AISa gave the second lowest docked binding energy. This molecule exhibited the second highest HIV-PR inhibition activity. Interestingly, the low energy docked cage peptide structures (PCU-EAISa and PCU-AISa) did not exhibit the same cage–peptide (CP) interaction observed with the NMR studies and the QM/MM/MD solution structures. This observation could suggest that the specific low energy CP conformation is not the active conformation, since the active enzyme pocket rearranges the cage peptide to the active conformation through an induced fit mechanism.

The crude docked binding energies largely follow the order of the *in vitro* HIV-PR IC_{50} results. Docking of the same inhibitors with C-SA PR where only one of the Asp25 residues was protonated, gave similar docking behavior to the unprotonated Asp25 residues. Though docking calculation could not provide deeper insight on the dynamic inhibitor-enzyme interactions, it provided us with a general picture on the energetically favorable binding orientation of inhibitors to the enzyme.

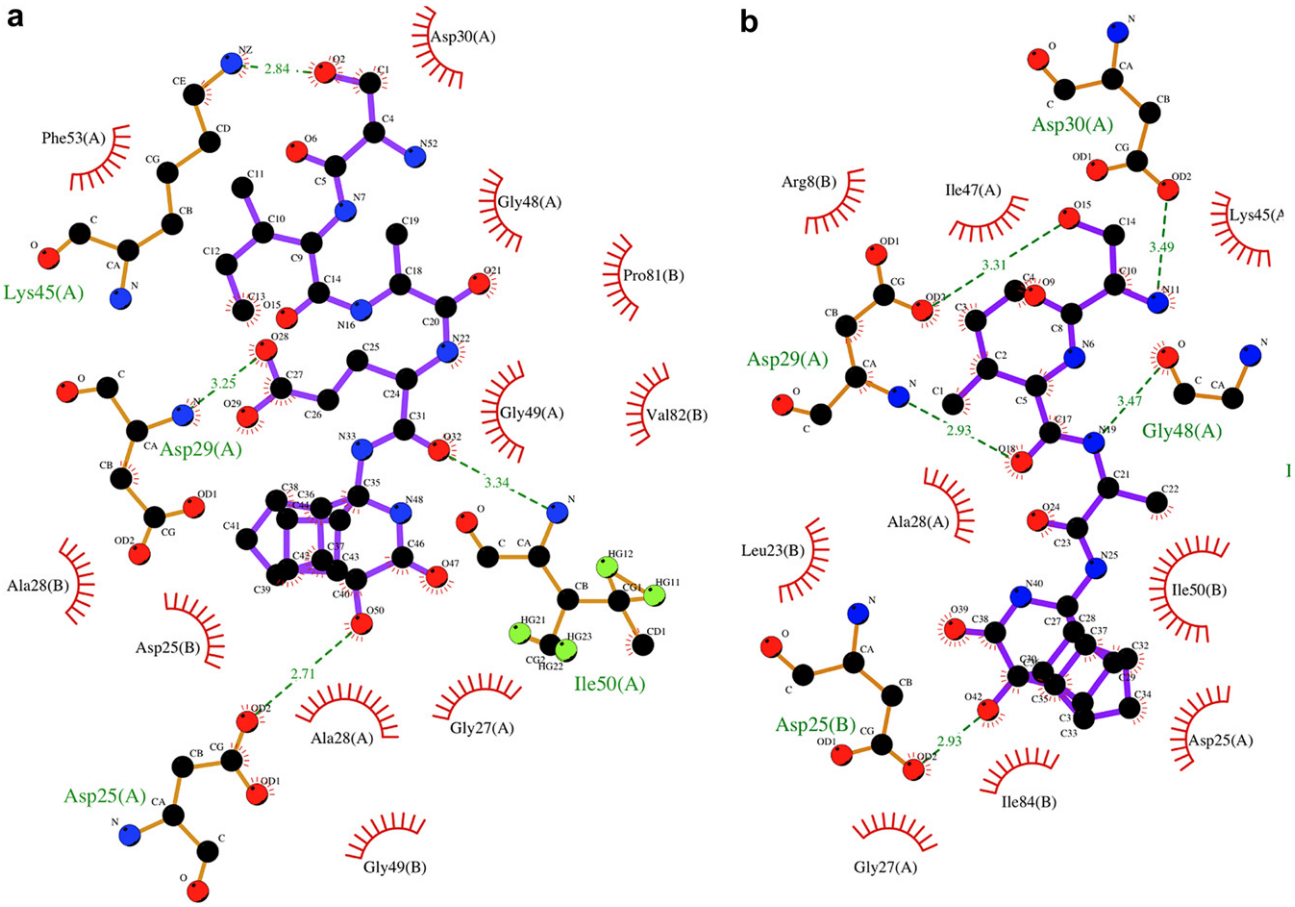

Fig. 6. modeled PCU protease interactions. Selected electrostatic and hydrogen bond interactions for the C-SA HIV-PR complexes with PCU-EAISa and PCU-AISa from the MD simulation. These plots were created with the Ligplot software [65]. (The plots for the electrostatic and hydrogen bond interactions for PCU-EAISb and PCU-AISb as well as the corresponding 3D structures for all complexes are provided in the Supplementary material.)

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3.2.3. QM/MM/MD simulation of the enzyme-inhibitor complex

To gain further insight into dynamic changes of the docked inhibitors inside the enzyme active site pocket over time, the lowest energy docked complexes of the inhibitors (PCU-EAISa/b and PCU-AISa/b) with the C-SA PR were subjected to unconstrained QM/MM/MD simulations (300 ps). This should provide a better simulated picture where full flexibility of the inhibitor and the enzyme is taken into account. The RMSD values obtained by averaging the trajectory over the 300 ps calculated for each complex relative to the initial minimized structures were calculated. These values were <1.2 Å. The overall orientation of the four complexes is preserved and very little movement of the cage lactam inside the enzyme pocket is observed during the course of the MD run. However, slight changes in the orientation in the inhibitor peptide side chain were observed (Fig. 6a and b).

Post-dynamic electrostatic and hydrogen bond interactions were plotted for the four complexes. As is evident from the analysis of the hydrogen bond interactions along the MD trajectories, PCU-EAISa and PCU-AISa formed hydrogen bonds between the cage hydroxyl group and at least one of the two Asp25 carboxyl groups. This interaction also exists for PCU-EAISb but did not develop during the MD simulation for the PCU–AISb complex. This is expected since the low energy docked complexes gave a starting structure where the cage hydroxyl group of PCU-AISb was rotated away from the two Asp25 residues and little movement of the change lactam was observed during the MD run for all four complexes. Based on the X-ray structures of inhibitor/HIV-PR (subtype B) complexes [49,63,64], it is clear that hydrogen bond interactions between the inhibitor and the Asp25 groups of the enzyme are crucial for effective binding and hence also for inhibitory activity.

The MD simulations clearly showed that all inhibitors easily fit into the active enzyme pocket. It also confirms that the chiral nature of the cage is responsible for different possible conformations. Cage diastereomeric peptides therefore exhibit vastly different HIV-PR inhibition activities.

4. Conclusion

The combination of the PCU-cage with HIV-PR substrate-peptides yields a potential lead compound for HIV-PR inhibition. From a series of seven cage peptides a promising IC_{50} of 78 nM was obtained. Variation of the amino acid sequence and protection of the cage hydroxyl group enabled us to determine a structure–activity relationship for this family of cage peptide inhibitors, showing that the cage with the natural HIV-protease substrate (PCU-EAIS) exhibits the best activity. These results indicate the importance of the cage for bioactivity: removal of the cage destroys activity, while activity is observed for cage peptides where the natural peptide sequence is markedly varied. The cage peptides are also several orders of magnitude less toxic to human MT-4 cells than Lopinavir. The EASY–ROESY technique has proven to be an extremely powerful method to provide vital information about the 3D solution structure of small peptides. All active inhibitors exhibited the same ROE interaction between the cage protons and the peptide side chain. NMR and QM/MM/MD simulations were instrumental in showing for the first time that the chirality of the PCU-cage induces different peptide conformations which are stable at temperatures up to 333K. The different cage-peptide diastereomers (PCU-AISa and b) were shown to exhibit a 20-fold difference in their *in vitro* IC_{50} PR inhibition capabilities. Protection of the cage hydroxyl group, which is proposed to be a norstatine type isosteric bond, resulted in a complete loss of inhibitory activity. This is in agreement with a docking experiment where the acetyl protected cage peptide appeared to have lost the required interaction between the norstatine bond and the two enzymatic Asp25 residues. The docking results also confirmed that most active cage inhibitor (PCU-EAIS), gives the lowest binding energy. An MD simulation revealed that the most potent compounds exhibited interaction between the cage hydroxyl group and one of the PR Asp25 groups. The computational results also suggest that the low energy CP conformation is most likely not the active conformation, as this conformation was not observed for the inhibitor-enzyme complexes. This conformation is likely to play a role in early enzyme-inhibitor recognition followed by an induced fit binding mechanism in which the conformation of the inhibitor changes to compliment the geometry of the enzyme active pocket. The combination of drug design with *in vitro* assays, NMR and computational techniques has enabled us to rationalize the observed HIV-PR inhibition for the different cage peptides. Employment of these techniques, combined with a systematic variation of alternative cage analogues and peptide sequences should lead to the design of more active cage peptide inhibitors in the future. The possibility that these cage peptides may inhibit alternative disease-related protease families will be investigated.

Acknowledgments

This research was supported by NRF (SA) TG (GUN: 66319), KP (GUN: 69728), CHPC (SA), HGK and PIA (SA-Sweden bilateral grant) and Aspen Pharmacare. The authors thank Prof. Jürgen Schleucher and Mr. Dilip Jagjivan for their assistance with the NMR experiments.

Appendix. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejmech.2011.05.071.

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