CHEMMEDCHEM

DOI: 10.1002/cmdc.200800390

Development and Characterization of Peptidic Fusion Inhibitors Derived from HIV-1 gp41 with Partial D-Amino Acid Substitutions

Fabrice Gaston,[a] Giovana C. Granados,[b] Sergio Madurga,[b, c] Francesc Rabanal,[b] Faouzi Lakhdar-Ghazal,[a] Ernest Giralt,[b] and Elmostafa Bahraoui*[a]

The aim of this study was to design synthetic peptides with D-amino acid substitutions that mimic the human immunodeficiency virus (HIV) gp41 HR2 region. The objective was to develop new and active C34 analogue peptides by introducing D-amino acid point substitutions at nonessential sites for HR1–HR2 interaction without disrupting the structure of the peptide. Herein we report a study with C34L peptide analogues, including the enantiomer peptide C34D, the retro-inverso analogue (RI), and two peptides with D-amino acid point substitutions (C34M2 and C34M3). Our results show that, with the exception of RI, these peptides adopt an α-helical structure and are, like C34L, able to interact with HR1, mimicked by the N36 peptide. Furthermore, we show that modifications introduced in C34M2, but not in C34M3, enhance its resistance to trypsin-mediated hydrolysis and increase the stability of C34M2 in physiological medium. Interestingly, our results show that C34 peptide analogues C34M2 and C34M3, but not C34D and its RI analogue, retain their ability to inhibit HIV-1 replication with an efficiency similar to that of the C34L peptide. These data underscore the interest in using D-amino acids at specific sites in the C34 peptide sequence and may lead to a new strategy for the development of more stable and active anti-HIV-1 peptidic drugs.

Introduction

HIV preferentially infects CD4 cells, including T4 lymphocytes, monocytes/macrophages, and dendritic cells.[1,2] This tropism is determined by the viral envelope glycoproteins, the cellular CD4 receptor, and CCR5 or CXCR4 co-receptors.[3] Envelope glycoproteins are synthesized as 160-kDa precursors that are cleaved at the trans-Golgi level by cellular endoproteases to yield the mature external envelope gp120 (also named surface unit [SU]) and the transmembrane (TM) gp41 glycoprotein.[4] Both proteins interact through noncovalent interactions to form trimers at the surface of viral particles and infected cells.[5] The high-affinity interaction between gp120 and CD4 ($K_d = 1$ nM) induces significant structural modifications that lead to the exposure of a new domain of gp120 that undergoes a second interaction with the chemokine receptors CCR5 or CXCR4.[3,6] Upon gp120 receptor and co-receptor binding, subsequent rearrangements within the TM gp41 lead to the exposure of a previously hidden hydrophobic N-terminal region known as the fusion peptide. The insertion of this hydrophobic N-terminal fusion domain into the membrane of the host cell then initiates fusion between the viral and host cell membranes.[7] This insertion is accompanied by structural modifications of the heptad repeat regions HR1 and HR2, which fold into hairpin-like structures: the two hydrophobic helices HR1 and HR2 adopt an antiparallel orientation to form a six-helix bundle (6HB). The latter structure can then promote fusion by bringing the viral and cellular membranes into close proximity.[8]

Three essential domains have been determined in the gp41 ectodomain. The N-terminal fusion peptide (FP; residues 512–527) is characterized by its hydrophobicity. HR1, a structured helical coiled-coil, also named N-HR (residues 542–592) is followed by HR2, also known as C-HR (residues 623–663), which is also a structured helical coiled-coil domain. This domain is important for the development of anti-HIV vaccines, as broadly neutralizing antibodies recognize an epitope in this region.[9] Despite the high variability of the HIV-1 genome, the HR1 and HR2 domains are conserved.[10] The relatively high sequence conservation of HR1 (~65% in the M group) relative to HR2 (34% in M group)[11] can be correlated with the localization of the Rev responsive element (RRE) within this region. This region is essential for the export of mono- and non-spliced viral mRNA from the nucleus to the cytoplasm.[12]

The fusion process is a complex phenomenon that depends on a wide range of structural, biochemical, and biophysical interactions. In addition, the molecular and structural mechanisms of membrane fusion, mediated by HIV-1 gp41, are still

[a] Dr. F. Gaston,[+] Dr. F. Lakhdar-Ghazal, Prof. E. Bahraoui
Laboratoire d'immuno-virologie
Université Paul Sabatier, UFR/SVT, 31062 Toulouse (France)
Fax: (+33) 561 558 667
E-mail: bahraoui@cict.fr

[b] Dr. G. C. Granados,[+] Dr. S. Madurga, Dr. F. Rabanal, Prof. E. Giralt
Institut de Recerca Biomèdica de Barcelona
Parc Cientific de Barcelona
Josep Samitier 1–5 08028 Barcelona (Spain)

[c] Dr. S. Madurga
Departament de Química Física de la Universitat de Barcelona-IQTUB
08028 Barcelona (Spain)

[+] These authors contributed equally to this work.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cmdc.200800390.

only partially understood, but enough to allow the development of new molecules that are able to block this key step of the viral cycle. To this end, the first applications showed that HIV-1 FP and its analogues[13,14] are able to block HIV-1 envelope glycoprotein-mediated fusion. So far, efforts have been focused on the development of fusion inhibitor peptides that interfere with HR1–HR2 interactions. T20 (DP-178, or enfuvirtide/Fuzeon) is a 36-residue peptide that shares two thirds of its N-terminal amino acid sequence with HR2.[15] This peptide efficiently inhibits HIV-1 replication in vitro at the nanomolar range[16,17] and in vivo.[18] Its inhibitory activity appears to be mediated by direct interactions with HR1 and by interaction of its C-terminal region, which is different from HR2, with the membrane or the transmembrane domain of gp41.[19–23] T20 is in current use as an entry inhibitor of HIV, primarily for patients who have developed resistance to the usual highly active antiretroviral therapy (HAART).[24] However, the emergence of resistance to T20 in new HIV-1 strains[25] requires the development of novel peptide-based inhibitors, including: 1) analogues of T20 such as T-1249,[26] a 39-residue, all-L-amino acid peptide composed of sequences derived from HIV-1, HIV-2, and simian immunodeficiency virus (SIV); the great advantage of this peptide is its ability to remain active against T20-resistant viruses.[27] 2) C34 peptide, corresponding to HR2, which inhibits HIV-1 infection in vitro at the nanomolar level; several analogues of HR2 regions have also proved to be potent inhibitors both in vitro and in vivo.[26,28,29] 3) N36 peptide, a 36 amino acid peptide corresponding to HR1; in contrast to T20 and C34, the N36 peptide inhibits HIV-1 with lower activity (IC_{50} in the micromolar range).[30] The use of the T20 peptide is also limited by its relatively short half-life in vivo.

The introduction of D-amino acids at specific positions may enhance the resistance of peptides or proteins to protease degradation. However, structural stability, conformation, and activity might be negatively affected.[31] The magnitude of the structural changes depends on the location and nature of the modification.[32] Partial D-amino acid substitutions could break tertiary structural integrity, thus decreasing peptide stability; in contrast, such substitutions could maintain a favorable peptide conformation with a lower cost to stability. For example, the substitution of glycine residues by D-alanine satisfies the topological requirements for retaining structure and function[33] or even increasing stability.[34,35] This suggests the possibility of substituting natural L-amino acids with their respective D-isomers without introducing significant perturbations to structure, and thus to the activity of the peptides or proteins.

The objective of this study was to initiate the development of new and active C34 analogue peptides by introducing D-amino acid point substitutions at the putatively more sensitive enzymatic cleavage sites, without disrupting the overall peptide structure. Herein we report our studies with C34L peptide analogues, including the enantiomeric C34D peptide, the retro-inverso analogue (RI), and two peptides with D-amino acid point substitutions (C34M2 and C34M3). Our results show that, with the exception of RI, these peptides adopt an α-helical structure, and are, like C34L, able to interact with HR1 mimicked by the N36 peptide. Furthermore, we show that modifications introduced in C34M2, but not in C34M3, enhance its resistance to trypsin-mediated hydrolysis and increase the stability of C34M2 in physiological medium. Interestingly, our results show that C34 peptide analogues C34M2 and C34M3, but not C34D and its RI analogue, retain their ability to inhibit HIV-1 replication with an efficiency similar to that of the C34L peptide.

Results

Design of C34 peptide analogues

Several aspects were taken into account in the rational design of the C34L analogues. First, because the use of D-amino acids could destabilize the helical tendency of C34, an approach using only limited residue substitution was chosen. Second, only C34 residues that are not important for the C34–N36 trimer interaction were selected for substitution. Third, mutated residues were selected in pairs that are separated by one turn of the C34 helix in order to promote the establishment of noncovalent bonds in the direction of the helix macro-dipole.

Calculations of the C34L peptide docked into the trimeric N36 three-helix core were performed to determine the most important residues in the C34–N36 interaction. Only one cluster of docked structures with an RMSD of 0.4 Å with respect to the crystallographic C34 binding mode was obtained, which indicates that a sufficient number of energy evaluations had been performed to obtain convergent results. The contribution of each C34L residue to the docking energy is shown in Figure 1. It is apparent that only a subset of residues make an important contribution to the stabilization of the C34 peptide within the N36 trimer core. The two major contributions correspond to Trp628 and Trp631, which are in a hydrophobic cavity formed inside the N36 core.[36] These two residues are in the N-terminal part of C34L and are separated by one turn. In the next three turns of the C34L helix, Ile635, Tyr638, and Ile642 are in contact with the N36 trimer, making an important contribution to the docking energy. However, for the last half of the C34 peptide, a different pattern of intermolecular interactions is observed. In general, at each turn the interaction involves more than one residue: Leu645 and Ile646 for the sixth turn; Glu648, Ser649, and Gln650 for the seventh turn; Gln652 and Gln653 for the eighth turn; Asn656 and Glu657 for the ninth turn; and finally, Leu660 for the last turn.

Accordingly, two C34L peptide analogues, C34M2 and C34M3, with different selected mutations were designed. For C34M2, two weakly interacting residues separated by one turn were selected in the N-terminal region (Glu654 and Gln658). The two C34 sequence changes were E654e and Q658k. We replaced Glu654 by its D-isomer to enhance the resistance of the cleavage site at Lys655. However, to conserve an ionic bond with its γ-COO^- group, the L-Gln residue at position 658 was substituted by D-Lys. As a consequence, the resulting ionic interaction was present at the opposite face of the helix, and helped to stabilize the α-helical structure of the peptide. For the second analogue (C34M3), in addition to the modifications introduced in the C34M2 peptide, two new modifications were

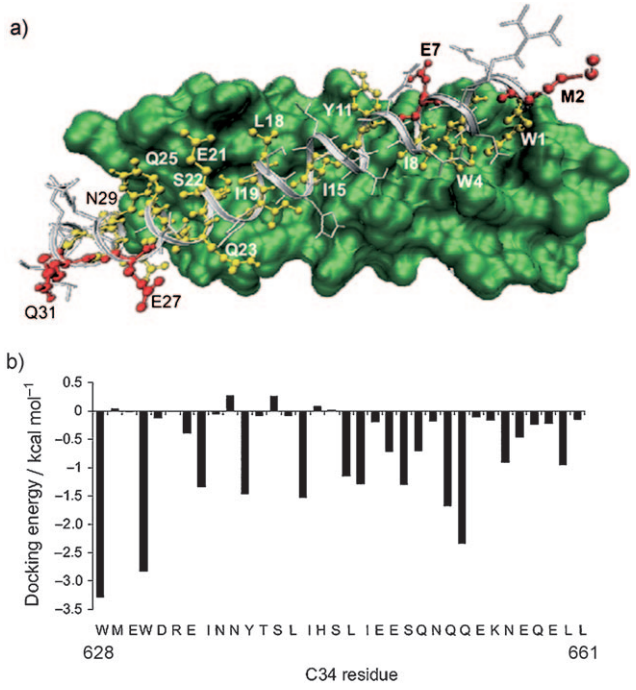

Figure 1. a) Representation of the C34L helix (gray) and two interacting N36 peptides (green) derived from PDB file 1AIK; yellow residues indicated positions directly involved in the interaction with N36 according to the docking calculations, and red residues show the positions which are substituted in the C34L analogues. b) Docking energy profile for all C34L residues calculated with AutoDock 4.

added. At position 629 L-Met was replaced by D-Glu, and at position 634, L-Glu was substituted by L-Lys. The goal of the first modification was to introduce a dipeptidyl protease-resistant site, and the aim of the second was to introduce a negatively charged amino acid able to establish a salt bridge with Lys634 in the direction of the macro-dipole. This modification attenuated the impact of introducing a D-amino acid at position 629. The docked structure of the C34 peptide in complex with the three-helix core of the N36 trimer is shown in Figure 1a. It can be seen that residues Met629 (M2 in the figure), Glu634 (E7), Glu654 (E27), and Gln658 (Q31) of C34L, which were changed to their respective D-isomers in C34M2 and C34M3, do not directly interact with the N36 trimer. Thus, the mutation of these residues does not disturb the formation of the C34–N36 complex to any significant extent, according to molecular dynamics calculations (see Supporting Information).

Physicochemical characterization of C34L and its analogues

The peptides were synthesized by using a solid-phase method with an Fmoc strategy. All the peptides were purified by HPLC and had a purity of at least 95% (Supporting Information). In addition to C34M2 and C34M3, native C34L, C34D (all D-amino acids), RIC34 (the retro-inverso analogue), C34M1 (a scrambled-sequence peptide), and N36 were also synthesized and used in order to compare the various interactions and activities. All peptides were acetylated and amidated at their N and C termini, respectively (Table 1).

Table 1. Amino acid sequences of synthesized peptides.

Peptide	Sequence[a]	Yield [%]
C34L	^{628}WMEWDREINNYTSLIHSLIEESQNQQEKNEQELL^{661}	17
C34M2	^{628}WMEWDREINNYTSLIHSLIEESQNQQeKNEkELL^{661}	26
C34M3	^{628}WeEWDRKINNYTSLIHSLIEESQNQQeKNEkELL^{661}	34
RIC34	^{628}lleqenkeqqnqseeilshilstynnierdwemw^{661}	ND[b]
C34D	^{628}wmewdreinnytslihslieesqnqqekneqell^{661}	17
C34M1	^{628}wedmwrnnvteylssilehveqqsnkqeeqenll^{661}	ND[b]
N36	^{546}SGIVQQQNNLLRAIEAQQHLLQLTVWGIKQLQARIL^{581}	24

[a] Upper-case letters denote L-amino acids; lower-case letters indicate D-amino acids. [b] Not determined.

The homogeneity of the peptides was confirmed by amino acid composition analysis and molecular weight determination by MALDI-TOF MS. The solubility of each peptide was calculated by MS as described in the Experimental Section. The concentrations of the peptides were: 11.3 μM for C34L, 70 μM for C34M2, and 131 μM for C34M3.

At a structural level, circular dichroism (CD) spectra for C34L, C34M2, and C34M3 alone in aqueous solution showed only moderate α-helical structure. In contrast, when the analyses were performed in 30% trifluoroethanol (TFE), the CD spectra clearly showed a double minimum at 208 and 222 nm, characteristic of the presence of an α-helical structure (Figure 2a–c). Interestingly, when the analysis was performed under the same conditions with the all-D-amino acid containing C34D peptide, a spectrum symmetrical to that of C34L was obtained (Figure 2a). This effect is related to the dextrorotatory D-amino acids of this peptide analogue.

CD spectral analysis was further used to study the capacity of C34 analogues to interact and form a 6HB (a trimer of hairpins)[8,37] with the N36 peptide. For that purpose, CD spectra were obtained with equimolar mixtures of N36 and C34 peptide analogues in aqueous solution. The corresponding CD spectra of these mixtures, shown in Figure 2, clearly show the capacity of C34L peptide and its analogues, C34M2 and C34M3, to interact with N36 peptide and to form α-helical structures. The relative degree of helicity of these complexes, listed in order of decreasing level, is N36+C34 (Figure 2d), N36+C34M3 (Figure 2f) and N36+C34M2 (Figure 2e). In contrast, no interaction was observed with the C34M1 peptide control (Figure 2g), as indicated by the absence of characteristic spectral features of α-helical structure.

The stability of the N36–C34 peptide interactions was further characterized by measuring the thermal denaturation values of the complexes formed between N36 and C34L, C34M2, or C34M3 peptides. The melting curves and the midpoint of the thermal unfolding transition (T_m) values were determined for each C34L peptide analogue (Figure 3). The complex between N36 and C34L had a T_m value of 62.7 °C, and those formed with C34M2 and C34M3 exhibited similar T_m values of 52.5 °C. These data show that, despite the respective introduction of two or three D-amino acids in C34M2 or C34M3, these peptides show greater solubility than C34L yet maintain their capacity to interact and form stable complexes with the N36 peptide.

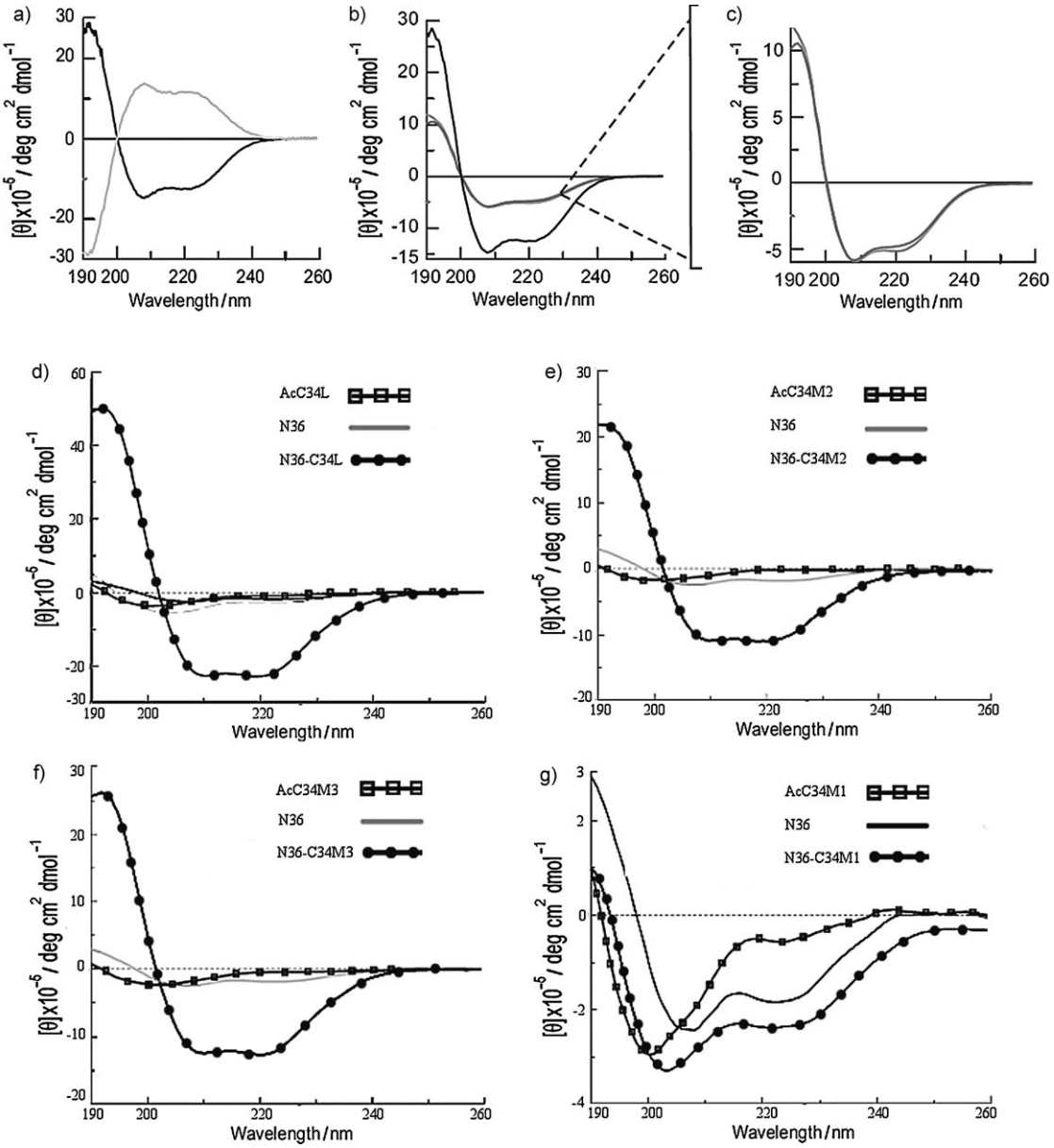

Figure 2. CD spectra of the various peptides and N36–C34 peptide complexes: a) C34L (black) and C34D (gray); b) C34L (black), C34M2 (light gray), and C34M3 (dark gray). Spectra were recorded at 25 °C with a peptide concentration of 56 μM in 30% TFE, 10 mM potassium phosphate, 50 mM potassium fluoride, pH 7.4. c) Enlargement of the C34M2 and C34M3 spectra. d–g) CD spectra of peptides in an equimolar mixture of d) N36+C34L, e) N36+C34M2, f) N36+C34M3, and g) N36+peptide control. Spectra were obtained in aqueous solution. The appearance of a maximum at 192 nm and a double minimum at 208 and 222 nm were used as indicators of peptide interactions and of the formation of α-helical structure.

Susceptibility of C34 peptide analogues to trypsin cleavage

Treatment of the C34L peptide with trypsin leads to the appearance of fragments resulting from cleavage at lysine or arginine residues. MALDI-TOF MS analysis allowed us to identify the peptide fragments that correspond to the hydrolysis of the Arg 633–Glu 634 and Lys 655–Asn 656 junctions. These products are in agreement with the peptides expected to result from cleavage at the basic residues Lys or Arg. The same approach was applied to the C34M2 peptide analogue. Analysis of the trypsin digest products by MALDI-TOF MS allowed us to identify only two peptides with the following sequences: WMEWDR and EINNYTSLIHSLIHSLIEESQNQQeKNEkELL. The absence of products resulting from hydrolysis between Lys 655 and Asn 656, previously identified from trypsin-treated C34L, can be easily explained by the introduction of a D-Glu residue near the potential cleavage site at Lys 655. Treatment of the C34M3 peptide analogue with trypsin under the same conditions showed a very rapid degradation of the peptide. MALDI-TOF MS analysis of the products allowed us to identify the products of hydrolysis at Arg 633–Lys 634 and Lys 634–Asn 635, which leads to the appearance of the following peptide fragments: WeEWDR, WeEWDRK, and RKINNYTSLIHSLIEESQNQQeKNEkELL. This high sensitivity of C34M3 toward trypsin digestion can be explained by the introduction of an additional trypsin cleavage

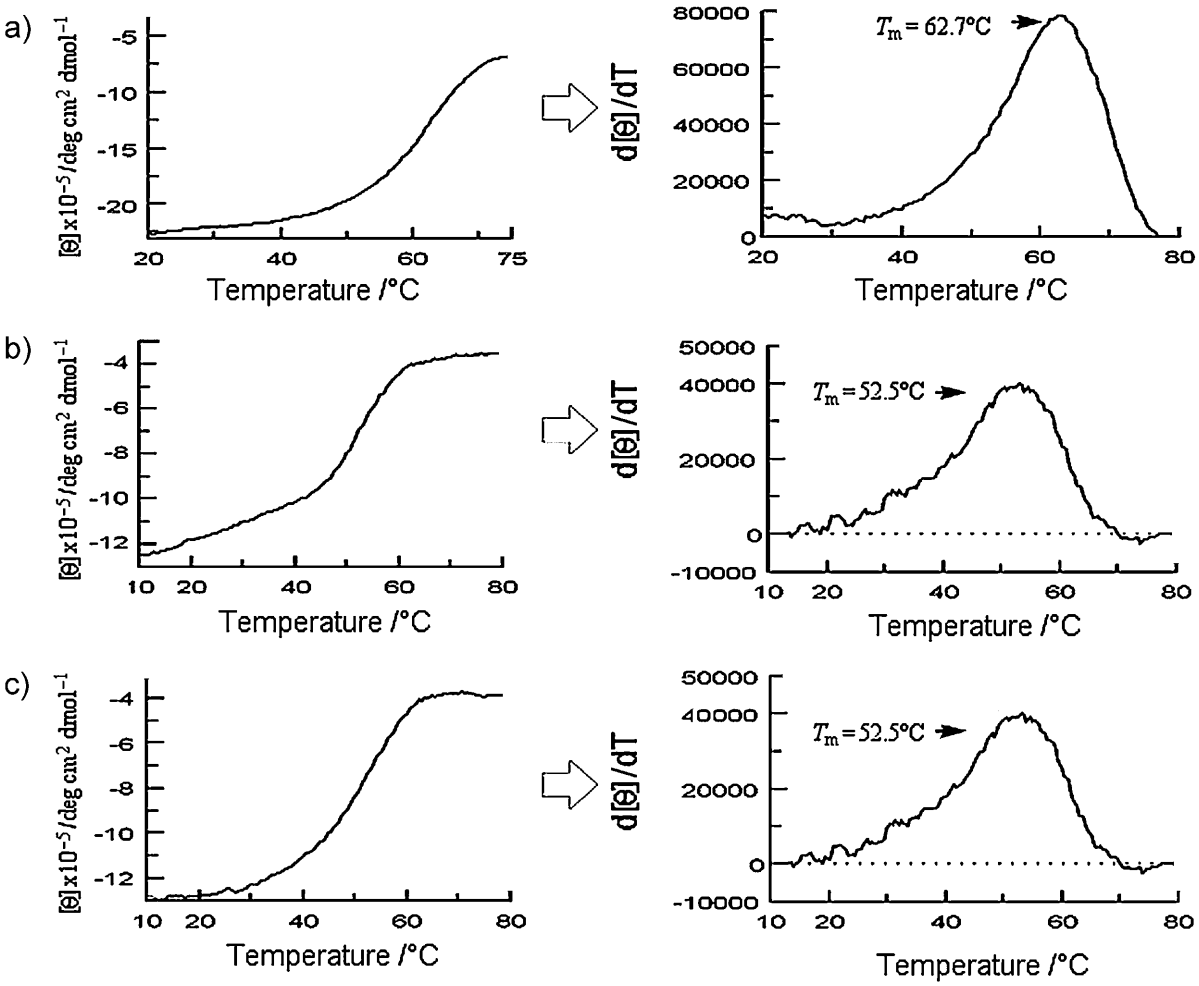

Figure 3. Thermal stability curves of the various N36–C34 complexes: complexes of N36 with a) C34L, b) C34M2, and c) C34M3 were obtained by monitoring the molar ellipticity at 222 nm of equimolar mixtures at 10 μM in 10 mM phosphate buffer, 10 mM potassium fluoride, pH 7.4. The plots at right show the first derivative of molar ellipticity, and the maxima indicate the melting temperature (T_m) in each case.

site by substituting L-Glu with an L-Lys residue at position 634. As expected, no cleavage products were observed with the same trypsin treatment of the all-D-configured C34D peptide, even after 48 h exposure (Figure 4).

Considering that the kinetics of hydrolysis are first order, the half-life of each peptide was determined by applying the formula: $\ln C = \ln C_0 - k_e t$, in which C_0 and C are the peptide concentrations at $t = 0$ min and at time t, respectively, and k_e is the experimental rate constant determined from the curves of degradation kinetics. The half-lives determined for the C34L peptide and its analogues are as follows: C34M2 $t_{1/2} = 133$ min, C34L $t_{1/2} = 78$ min, and C34M3 $t_{1/2} < 15$ min (Figure 4). These data clearly underscore the positive or negative effect of the mutations introduced in C34M2 or C34M3, respectively, with respect to their stability toward trypsin treatment.

Using the same approach, C34L and its analogues were subjected to chymotrypsin digestion for 15, 30, 60, and 120 min before HPLC analysis. The results show the relative sensitivity of C34L, C34M2, and C34M3 to chymotrypsin digestion (Figure 5). Peptide cleavage reached ~50% in the following order: C34L 15 min, C34M2 35 min, and C34M3 40 min. The

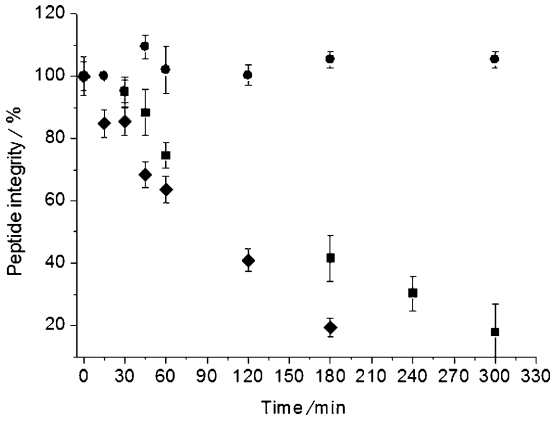

Figure 4. Kinetics of trypsin-mediated peptide degradation: peptides C34L (◆), C34M2 (■), and C34D (●) were incubated with trypsin for various amounts of time, and the digestion products were then separated and quantified by RP-HPLC. For each peptide, the percentage of peptide integrity is reported versus the time of trypsin digestion; $t_{1/2}$ values were calculated as described in the Experimental Section. Because of its rapid degradation ($t_{1/2} = 15$ min), data for C34M3 are not shown here.

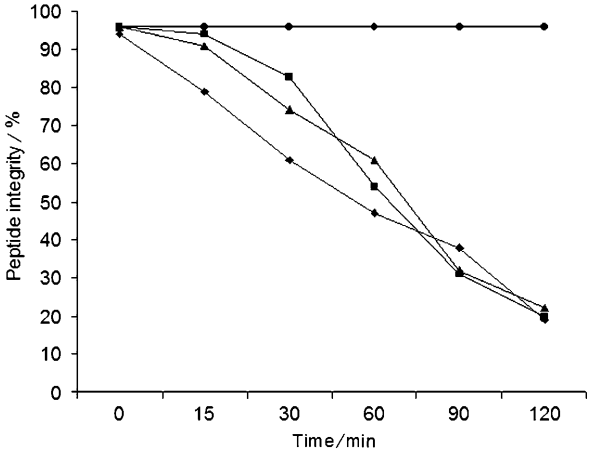

Figure 5. Kinetics of peptide degradation by chymotrypsin: for each peptide, C34L (◆), C34M2 (■), C34M3 (▲), and C34D (●), the percentage of peptide integrity is reported versus the time of chymotrypsin treatment.

high sensitivity of these peptides to chymotrypsin digestion is due to the high number (>12) of potential cleavage sites. Here again, no cleavage was observed in the all-D-amino acids containing peptide C34D.

Anti-HIV activities of C34 and its analogues

The antiviral activities of C34 and its analogues were investigated in three complementary assays including the inhibition of: 1) cell–cell fusion, 2) single-cycle viral replication, and 3) quantitative viral replication. Before using peptides on cells, their eventual intrinsic cytotoxicity was evaluated. Peptides at concentrations up to 10^{-5} M in culture with HeLa or primary cells did not exhibit any observable cytotoxicity as evaluated by trypan blue exclusion assay or by comparing the growth curves of cells in the presence or absence of various peptide concentrations (10^{-5}–10^{-9} M; data not shown).

1. The potency of C34L and its analogues to inhibit cell–cell fusion

The capacity of the C34L peptide and its analogues to block syncytium formation between HeLa cells expressing envelope glycoproteins from HIV-1 LAI (X4 tropic virus), or HIV-1 ADA (an R5 macrophage tropic virus) and HeLa cells expressing the human CD4 receptor CXCR4 or CCR5 HIV-1 co-receptors was investigated in the presence of various amounts of each peptide. Results from these experiments, shown in Figure 6a,b and Table 2, show that C34L, C34M2, and C34M3 inhibit syncytium formation in a dose-dependent manner. Potent and nearly complete inhibition was observed at 10^{-7} M with all the three peptides. The concentrations at which syncytium formation is inhibited by 50% (IC_{50}) were determined for each peptide, and are listed in Table 2. The nanomolar IC_{50} values show that, despite the introduction of non-natural D-amino acids at some positions, the high antiviral activities of the peptides are conserved. However, no antiviral activity was observed with the all-D-amino acid containing C34D peptide or with the RI analogue (Figure 6).

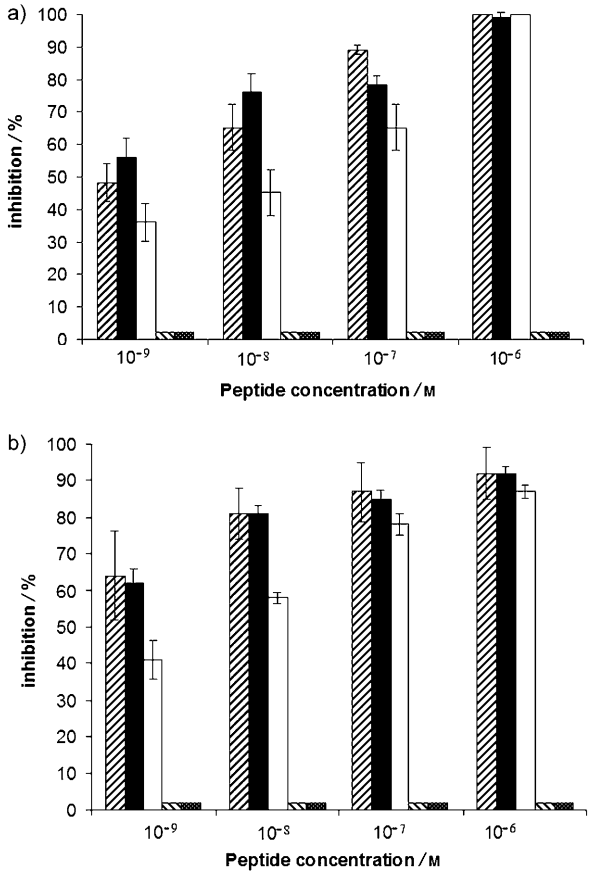

Figure 6. Inhibition of syncytium formation in the presence of C34L and its analogues: a) HeLa gp120/gp41 ADA or b) HeLa gp120/gp41 LAI cells were co-cultivated with HeLa-P4P5 in 96-well plates in the presence of various concentrations of peptides C34L (upward hash), C34M2 (black), C34M3 (white), C34D (downward hash), and RI (gray). After co-culture for 20 h, syncytia were scored by optical microscopy.

Table 2. Anti-HIV-1 activities of C34L and its analogues determined by HIV-1 replication and syncytium assays.

	Inhibition of HIV-1 replication[a] IC_{50} [nM][b]			Inhibition of syncytium formation	
Peptide	BaL	VN44	LAI	ADA	Loss of activity [%][c]
C34L	2.65	0.58	0.41	2	70
C34M2	2.53	0.31	0.15	0.3	38
C34M3	5.57	5.79	4	5.8	100
C34D	--	--	--	--	–

[a] At day 3 post-infection. [b] IC_{50} values correspond to peptide concentrations that inhibit HIV-1 replication or syncytium formation by 50%. [c] After 48 h incubation in human serum.

2. Antiviral activities of C34 and its analogues in a single viral infectivity assay

In this assay, C34 and its analogues were tested at various concentrations for their capacity to inhibit the infection of HeLa

CD4-CCR5/CXCR4-LTR/β-Gal cells with HIV-1 LAI or HIV-1 BaL over a period of 20 h, a time corresponding to a single viral cycle. In fact, this assay enabled us to monitor the completion of the first steps of the viral cycle, including adsorption, penetration, and early genome expression. The assay was based on the ability of the early produced Tat protein to trans-activate the expression of *LacZ* gene under the control of HIV promoter. In agreement with the cell–cell fusion assay, inhibition of cell entry was observed with the C34L peptide and its C34M2 and C34M3 analogues, whereas no inhibition was observed with the C34D or RI peptides (Figure 7, Table 2). Interestingly,

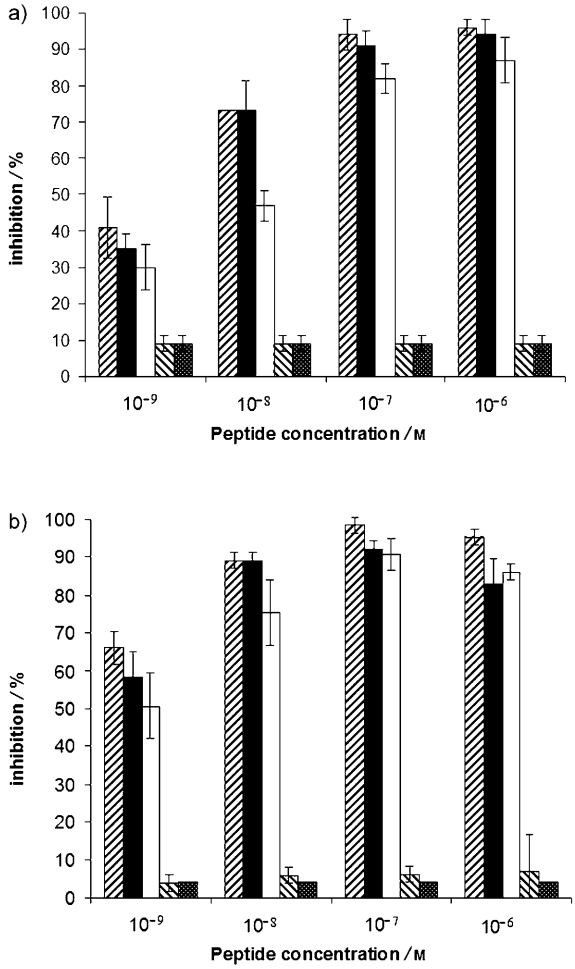

Figure 7. Anti-HIV-1 activity of C34L and its analogues in a single infectivity assay: HeLa CD4-CCR5/CXCR4-LTR/β-Gal cells were infected for one viral cycle (20 h) with 0.1 ng a) HIV-1 LAI or b) BaL as described in the Experimental Section in the presence of peptides C34L (upward hash), C34M2 (black), C34M3 (white), C34D (downward hash), and RI (gray). Viral replication was correlated directly with the transactivation of the *LacZ* gene by the early translated HIV-1 *Tat* gene. β-Galactosidase activity was visualized by incubating cells with the substrate X-Gal, which stains cells blue after being degraded.

C34L and its analogues seemed to be more active against the macrophage tropic than the T tropic virus in this assay (Figure 7).

3. Antiviral activities of peptides by quantitative assay

In contrast to the two previous assays, primary PBL cells or macrophages were used as target cells and infected with a T tropic virus (HIV-1 VN44) or macrophage tropic virus (HIV-1 BaL). The virions produced were quantified in the cell supernatants after three days of infection. The results show that C34L and its analogues fully inhibit HIV-1 VN44 and HIV-1 BaL replication at respective concentrations of 10^{-6} M and 10^{-7} M (Figure 8, Table 2). The IC_{50} values listed in Table 2 show that

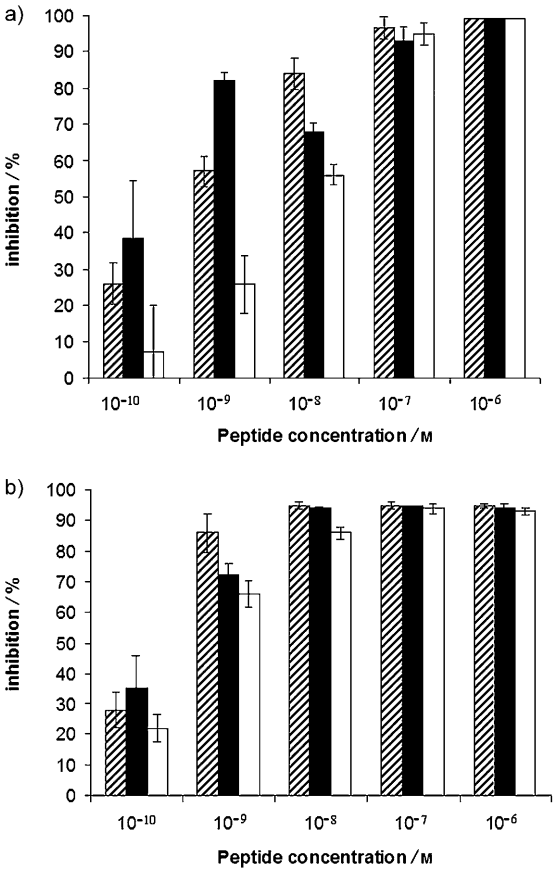

Figure 8. Effect of C34L and its analogues on viral replication of X4 and R5 tropic viruses: a) lymphocytes or b) macrophages were infected with 0.3 ng HIV VN44 (X4 tropic) or BaL (R5 tropic), respectively, for 3 h as described in the Experimental Section in the presence of peptides C34L (upward hash), C34M2 (black), and C34M3 (white). Three days post-infection, cell supernatants were collected and tested by ELISA for the p24 antigen. Uninfected cells treated in the same way were used as control. No inhibition was observed with C34D or RI (data not shown).

C34 and its analogues exhibit similar potency to inhibit HIV-1 replication, with IC_{50} values in the nanomolar range (0.3–5.8 nM). No antiviral activity was observed with the C34D or RI analogues.

Overall, our data show that the inhibitory activity of the C34 peptide is chirality dependent for the all-D-amino acid containing C34D analogue, and chirality independent for cases in which only some D-amino acids are present in the peptide, as shown with C34M2 and C34M3.

Comparison of the stability of C34 and its analogues in human serum

To test the stability of the antiviral activity of C34L and its analogues in human serum, peptides were incubated for various periods of time in human serum before determining the percent conservation of their initial antiviral activities. As a control, peptides or sera alone were tested in the same assay. The results show that C34M2 has the highest stability relative to C34L and C34M3 (Figure 9). After 48 h incubation, antiviral activity losses of 100, 70, and 38% were observed with C34M3, C34L, and C34M2, respectively (Figure 9, Table 2). Notably, these results correlate with the relative protease resistance profiles for these peptides.

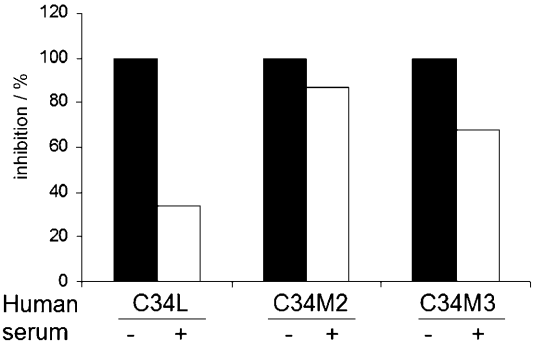

Figure 9. Stabilities of C34L, C34M2, and C34M3 after incubation in human serum: peptides were incubated for 24 h in human serum and then tested for their capacity to block syncytia formation between HeLa gp120/gp41 and HeLa-P4P5. White bars indicate remaining antiviral activities; black bars correspond to the antiviral activities of sera-untreated peptides.

Discussion

All HIV inhibitors, unfortunately, face the problem of emerging viral resistance. To circumvent this problem, it is important to develop fusion inhibitors that bind key regions in the gp41 HR1 and HR2 domains in different ways to offset the potential for cross-resistance among agents in the fusion inhibitor class. For example, biochemical studies of HR2-derived peptides have shown that the molecular mechanisms involved in the inhibition of HIV fusion by C34 and T20 are different despite the fact that both are efficient in the nanomolar range.[38] As for the C34 peptide, T20 antiviral effect is also mediated by its interaction with HR1, but at different sites from those engaged with C34. In fact, T20 overlaps with C34 only by its first 23 N-terminal amino acid residues. Data from X-ray diffraction studies of HR1–HR2 complexes implicate Trp628, Trp631, and Ile635 (absent in the T20 sequence) in interaction with residues Val570, Lys574, Gln577, Leu568, Trp571, Gly572, Thr569, Ile573, and Leu576 of the conserved pocket of HR1.[37] These interactions have been shown to be important for the stabilization of the 6HB.[38] The most active analogues of C34 peptides, including T-1249 and T-649, contain these three essential amino acids. Interestingly, the high efficiency of T-649, an analogue with a two-amino acid extension at the C-terminal end of C34, against a panel of T20-resistant HIV-1 primary isolates seems to be associated with the higher stability of the 6HB formed by this peptide.[38] T-1249, an analogue of both T-649 and T20, also showed antiviral activity against T20-resistant primary isolates.[39] However, the development of such peptides has remained limited by problems related to their solubility and formulation.[27] Therefore, any approach that leads to an enhancement of the correct peptide folding, stability, and solubility needs to be investigated.[40–42]

For example, despite its high antiviral activity, the L-amino acid containing peptide T20 (enfuvirtide, Fuzeon) suffers from several limitations, including delivery by injection, large dose requirements (90 mg twice daily), emergence of resistant isolates, and high cost. These constraints explain why this peptide is used particularly for the treatment of patients with HIV infections that have developed resistance to reverse transcriptase and protease inhibitors. To circumvent some of these limitations, synthetic peptides with D-amino acids need to be developed further. The first study using this approach described a 16-mer peptide that had only modest antiviral activity (IC_{50} = 10 μM).[43] Recently, however, another study has shown that dimers or trimers of a D-amino acid containing 15-mer peptide, also screened by a mirror-image phage display approach, interact with HR1 with high affinity and are highly efficient at inhibiting HIV-1 replication (IC_{50} = 250 pM).[44] Interestingly, this peptide, except for the conservation of the WXWL consensus sequence, has no evident sequence homology with the C34L peptide. The concept of its design was based essentially on a peptidomimetic approach to screen molecules that are able to bind to the N-trimer pocket with high affinity and to inhibit HIV-1 penetration. Analysis of the crystal structure of this potent D-peptide inhibitor of HIV-1 in complex with the HR1 domain revealed several key interactions, including D-Tyr7–Trp571, D-Lys2–Trp571, and D-Tyr7–Glu575, which explain its high anti-HIV potency.[44]

Because several limitations of L-peptides, including T20, are related to their sensitivity to protease activity, D-peptides have many theoretical advantages. In the study presented herein, we have shown that, as expected, the C34D peptide shows great resistance to protease treatment. It remains uncleaved even after 48 h incubation with trypsin or chymotrypsin. However, despite the fact that it retains the ability to adopt an α-helical structure, this peptide is inactive as an antiviral agent. This lack of antiviral activity correlates with its inability to interact with the N36 peptide, as analyzed by CD (data not shown). At the structural level, this lack of antiviral activity may be explained by the fact that the highly conserved residues, mostly residing at the a, d, and e positions in the heptad structure, are further oriented toward the opposite face of the conserved hydrophobic pocket of HR1. As a result, no contact between C34D and the pocket structure of HR1 can occur. To try to compensate for these structural disadvantages we synthesized the retro-inverso (RI) analogue of C34L. This type of modification is accomplished by simply reversing the peptide sequence direction and inverting the chirality of each amino acid. Several studies have reported that retro-inverso peptides are topologically correlated with the parent peptide, as the resulting side

chain dispositions are similar to those of the parent peptide, but carbonyl and amine groups are interconverted from their positions. Unfortunately, this RI peptide does not retain antiviral activity; this may be related to the importance of the helical structure. It has been reported that the folding of the RI peptide may be hindered if the peptide contains helical segments.[45] However, several studies have shown that, in some cases, RI peptides can retain their biological activity with increased stability, as reported for enkephalin, glutathione, substance P, gastrin, and atrial natriuretic peptide.[46]

As an alternative, we modified our strategy in order to follow the antiviral activity of our designed peptide inhibitor step by step. We synthesized diastereomeric analogues of the C34L peptide corresponding to the HIV-1 gp41 HR2 domain with two, three, or all residues replaced by D-amino acids. Our objective was to design peptides that have: 1) the capacity to interact with the HIV-1 gp41 HR1 domain, 2) a propensity to adopt a helical structure, 3) increased stability in the presence of protease activity, and 4) antiviral activity.

Synthesized C34L, C34M2, and C34M3 peptides were purified to homogeneity. Their amino acid analysis after acid hydrolysis gave values consistent with the expected amino acid composition and were in agreement with MALDI-TOF MS analysis. Structural characterizations of these peptides by CD revealed their propensity to adopt α-helical structures, as shown by the presence of double minima at 208 and 222 nm in their respective spectra. Notably, the CD spectrum in TFE of the all-D-amino acid containing peptide C34D showed an α-helical structure with a mirror-image ellipticity profile at 208 and 222 nm relative to the spectra obtained with C34L, C34M2, and C34M3. This observation indicates that the α-helical structure of C34L seems to be chirality independent. Interestingly, the α-helical structure of C34 peptide analogues is observed only when the CD analysis is performed in TFE, which is an nonpolar helix-promoting solvent. However, even in aqueous solution, CD analysis of C34L, C34M2, and C34M3 performed in the presence of an equimolar amount of N36 peptide corresponding to the HR1 domain clearly showed an α-helical structure for each complex. These induced-order structures indicate that the N36 peptide is able to interact with C34L, C34M2, and C34M3, presumably to form a six helix bundle (6HB). This interpretation is in agreement with our data showing the capacity of C34L and N36 peptides to form oligomers in SDS-PAGE experiments, corresponding to an association of trimers of C34L and N36 peptides (data not shown). Furthermore, we showed that modifications introduced in C34M2, but not in C34M3, enhanced its stability in a physiological medium, as demonstrated in human serum, as well as its resistance to trypsin treatment. C34M2 has a half-life of 133 min, which is about twice that of the C34L peptide ($t_{1/2} = 78$ min). In human serum, C34M2 shows twice the stability of C34L. This stability enhancement can be explained by the introduction of D-amino acids at a trypsin-sensitive cleavage site (after Lys 655). In contrast, additional modifications, specifically the presence of L-Lys at position 634 in place of L-Glu, led to a rapid weakening of the C34M3 peptide, as shown by its short half-life of less than 15 min. Taking the latter result into account, synthesis of the same peptide with D-Lys at position 634 is currently underway.

All C34 peptide analogues except C34D and RI were able to block HIV-1 replication of both CCR5 and CXCR4 tropic viruses. These inhibition events were mediated by blocking virus penetration, as suggested by their capacities to inhibit syncytium formation. The data obtained with C34M2 are very promising, as they show that the introduction of D-amino acids at some critical sites 1) enhance the stability of the peptide in human sera, 2) increase peptide resistance to trypsin cleavage, 3) enhance the solubility of this peptide, and 4) do not interfere with the antiviral activity of the peptide, which remained active with IC_{50} values in the nanomolar range. The improved solubility of C34M2 (70 μM) relative to C34L (11 μM) makes this peptide worthy of consideration for clinical development. This point is critical because peptides with high antiviral activity yet low solubility are incompatible with clinical use, as is the case for T-1249 and T-649.[27] Similarly, the generation of peptides containing D-amino acids, but with low antiviral activity, remains unattractive for clinical application, as was the case for the first D-peptide reported by Eckert et al., which inhibited HIV entry with modest potency (IC_{50}: 10 μM).[43] However, more recent data reported by Welch[44] and Lee[47] show that partial or total D-amino acid substitutions can also generate molecules with strong antiviral activities. Therefore, it would be interesting to introduce the same modification as that performed by Lee et al. into our C34M2 peptide.

Overall, our data show that partial D-amino acid substitutions in the C34 sequence, if correctly placed at certain critical sites, can help to increase the stability of the peptides without altering their antiviral activity. Our objective is to continue to develop this approach in the design of new peptides with efficient antiviral activity and without the limitation of the T20 peptide. A great challenge for future studies will be to develop peptide-based inhibitors that can interact with the HR1 domain with high affinity while maintaining high stability and solubility.

Experimental Section

Docking calculations: The gp41 ectodomain structures were selected for calculation of docking of the C34L peptide into the N36 peptide trimer. The structure was retrieved from the Protein Data Bank (PDB code: 1AIK).[37] AutoDock Tools were used to assign Gasteiger partial charges to C34L and N36 peptides and to construct a grid map of $90 \times 90 \times 126$ points with a grid-point spacing of 0.420 Å. The maps were centered on the C34L–N36 contact surface. AutoGrid 4.0 was used to calculate the grid maps. Rigid docking calculations were performed with AutoDock 4[48] using the known crystallographic conformation of the C34L peptide for the ligand coordinates; 200 dockings were performed with the Lamarckian genetic algorithm using a population size of 50 individuals with a total of 5×10^7 energy evaluations. The crystallographic coordinates of the C34L–N36 complex were used as the reference structure to evaluate the predicted docked structures.

Peptide synthesis: Peptides were synthesized automatically in parallel on Rink amide-Gly-MBHA resin (330 mg) with low loading (0.3 mmol g^{-1}) on an Applied Biosystems 433A peptide synthesizer

by using Fmoc chemistry. The MBHA resin HL (loading: 0.77 mmol g^{-1}, Novabiochem) was solvated by successive washing steps with CH_2Cl_2 (3×1 min), 10% TFA in CH_2Cl_2 (1×10 min), 1% DIEA (3×1 min), CH_2Cl_2 (3×1 min), and *N*,*N*-dimethylformamide (DMF; 3×1). Fmoc-Gly-OH (0.1 mmol), pre-activated with *O*-benzotriazol-1-yl-*N*-tetramethyluronium tetrafluoroborate (TBTU)/1-hydroxybenzotriazole (HOBt)/*N*,*N*-diisopropylethylamine (DIEA) (1:1:1.5) in DMF, was coupled. After 2 h, the resin was washed with DMF (3×1 min) and CH_2Cl_2 (3×1 min), and the free amino groups were capped with Ac_2O/DIEA/DMF (3:1:16) (2×30 min). The resin was washed with DMF (3×1 min), and the Fmoc group was removed with 50% piperidine/DMF (3×5 min). Rink amide linker was coupled after pre-activation with TBTU/HOBt/DIEA (1:1:1.5) in DMF. Fmoc/*t*Bu-protected amino acids (Iris Biotech GmbH) were incorporated using standard procedures and TBTU/HOBt/DIEA as coupling agents. The Fmoc group was removed by treatment with 22% piperidine and 0.07% Triton X-100 in DMF. After final deprotection, peptides were acetylated by treatment with Ac_2O (40 equiv) and DIEA (9 equiv) in DMF. Concomitant side chain deprotection and cleavage was performed by treatment with a mixture of trifluoroacetic acid (TFA)/H_2O/triisopropylsilane/ethanedithiol (92:4:2:2 *v*/*v*/*v*/*v*, 5 mL) at 0 °C for 30 min and at room temperature for 1.5 h. TFA was removed by evaporation, and the crude peptide was precipitated with *tert*-butylmethyl ether. The products were characterized by HPLC, amino acid analysis, and MALDI-TOF mass spectrometry.

Synthesis was carried out with either L- or D-amino acids, and the sequences are listed in Table 1. In all cases, purified peptides were obtained after synthesis on Rink amide-MBHA resin (0.03 mmol). Purification was performed on an initial gradient of 10→30% CH_3CN with 0.5% TFA for 5 min, followed by linear gradients of 30→60% (AcC34L), 30→47% (AcC34D), 35→55% (AcC34M2), and 30→50% (AcC34M3 and N36) of CH_3CN with 0.5% TFA for 20 min at a flow rate of 10 mL min^{-1}; UV detection was performed at 220 nm.

HPLC analysis: The purity of the peptides was analyzed by analytical reversed-phase (RP) HPLC using a Symmetry C_{18} column (3.9 mm×150 mm, Waters) with a particle size of 5 μm and a pore size of 100 Å. The peptides were purified with a Symmetry C_{18} column (30 mm×100 mm, Waters) with a particle size of 5 μm. Flow rates (1 and 10 mL min^{-1}) were used for analytical and semi-preparative scales, respectively. Detection was performed at 220 nm with a UV detector. Solvent system A: 0.045% TFA in H_2O for analytical HPLC and 0.1% TFA in H_2O for semi-preparative HPLC; solvent system B: 0.036% TFA in CH_3CN for analytical HPLC and 0.1% TFA in CH_3CN for semi-preparative HPLC.

Circular dichroism: Spectra were recorded on a JASCO J-810 polarimeter equipped with a CDF-426S Peltier unit and a teflon-stoppered quartz cuvette with an optical path length of 0.1 cm. The peptide solutions were dissolved in phosphate buffer (10 mM potassium phosphate, 50 mM potassium fluoride, pH 7.4). Peptides at 56 μM were dissolved in aqueous buffer or in the presence of 30% trifluoroethanol (TFE). All analogues were soluble in these buffers at this concentration except C34L and C34D. The N36 peptide aggregated at concentrations >10 μM. The equimolar mixture of N36 and C34 analogues were previously dissolved in ammonium carbonate (100 mM, pH 8.0), neutralized with CH_3CO_2H (1 M) and lyophilized. The dry samples were dissolved in phosphate buffer at pH 7.4 (10 μM). Measurements were performed in the range λ = 190–260 nm with a data pitch of 0.2 nm, a band width of 1 nm, and a scanning speed of 20 nm min^{-1} for three accumulations. All spectra were corrected by subtracting the spectrum of buffer alone. The CD data were expressed as the molar ellipticity $[\theta]$ (deg cm^2 $dmol^{-1}$), calculated by:[49,50]

$$[\theta] = \frac{\theta}{10\,C\,l}$$

in which θ is the ellipticity (in mdeg), C is the molar concentration, and l is the path length of the cell in cm. Experiments on the thermal stability of the equimolar mixtures were performed at a constant heating rate of 90 °C h^{-1} and a response time of 1 s. The measurements were acquired in the range of 10–80 °C with a data pitch of 0.5 °C, monitoring the ellipticity at 222 nm. Thermal denaturation experiments were repeated twice with new samples.

Enzymatic hydrolysis: Each peptide was dissolved (up to 100 μM) in ammonium bicarbonate buffer (50 mM, pH 8.5). Trypsin (Promega, MS grade) was added at a molar peptide/trypsin ratio of 1:1500. Hydrolysis was performed by incubation at 37 °C. To monitor the reaction, aliquots (50 μL) were harvested at various time points between 0 and 5 h, and the last aliquot was taken 48 h later. The reaction was stopped by adding CH_3CO_2H (150 μL, 1 M), and the sample was immediately stored at 20 °C or processed for MS and chromatography analysis. A similar protocol was used for chymotrypsin digestion. Each aliquot was analyzed by RP-HPLC using a Symmetry C_{18} column and MALDI-TOF MS.[51] The diluted samples were eluted with a linear gradient of 0→70% CH_3CN (with 0.036% TFA in CH_3CN and 0.045% TFA in H_2O). The relative amount of nonhydrolyzed peptide was determined from the peak area. Considering that the enzymatic reaction follows first-order kinetics:

$$\ln C = \ln C_0 - k_e t$$

in which C is the concentration, which is proportional to the HPLC peak area expressed as percentage, C_0 is the initial concentration, k_e is the experimental rate constant, and t is time (min). The half-life ($t_{1/2}$) was calculated from the k_e value obtained using the following relation:

$$t_{1/2} = \frac{\ln 2}{k_e}$$

Viruses: The X4 HIV-1 LAI strain was obtained from Diagnostic Pasteur (Marne la Coquette, France) as a supernatant containing 10^5 $TCID_{50}$ mL^{-1}. The R5 HIV-1 BaL and VN44 X4 were obtained from Dr H. Hocini (ANRS, France). These viruses were amplified on monocytes derived macrophages or lymphocytes. The virus harvested at day 7 post-infection was titrated, separated into aliquots (100 ng p24 per mL) and stored at −80 °C until use.

Isolation of monocytes and differentiation in macrophages: Peripheral blood mononuclear cells (PBMC) from the buffy coats of healthy HIV-negative donors were isolated in a ficoll density gradient (Amersham Bioscience, Uppsala, Sweden). The cells were then plated at a density of 5×10^6 cells per well in 24-well tissue culture plates and maintained at 37 °C in an atmosphere of 5% CO_2. After 45 min, nonadherent cells were removed, and the remaining cells were washed three times with Hank's buffered salt solution and incubated in medium containing 90% Iscove-modified Dulbecco's medium, 10% fetal calf serum (FCS), 1% macrophage colony-stimulating factor, and a 1% penicillin–streptomycin mixture. The medium was changed every three days. On day 7, differentiated macrophages were infected with HIV-1.

Cell lines: HeLa CD4-CCR5/CXCR4-LTR/β-Gal cells, a gift from Dr. P. Charneau (Pasteur Institute, Paris, France), are HeLa cells that stably express human CD4, human CCR5, or CXCR4 co-receptors and contain the *LacZ* gene under the control of the HIV-1 LTR promoter. HeLa gp120/gp41 LAI or HeLa gp120/gp41 ADA are HeLa cells stably expressing gp160 from HIV-1 LAI or HIV-1 ADA isolates.

They were grown in complete Dulbecco's modified Eagle's medium in the presence of geneticin (1 mg mL^{-1}) for HeLa CD4-LTR/β-Gal or 1 μM methotrexate for HeLa gp120/gp41 LAI and HeLa gp120/gp41 ADA.

Syncytium formation: The synthesized C34 peptide analogues were tested at various concentrations (10^{-6} M–10^{-11} M) for their ability to inhibit syncytium. HeLa CD4-CCR5 or HeLa CD4-CXCR4 (10000 cells) were co-cultured with HeLa gp120/gp41 LAI or HeLa gp120/gp41 ADA (10000 cells) in 96-well plates in the presence of various concentrations of each peptide. After 20 h, syncytia were scored by contrast phase microscopy.

β-Gal test: Cells were washed twice with phosphate-buffered saline (PBS; 0.5 mM $MgCl_2$, 1 mM $CaCl_2$), fixed with 0.5% glutaraldehyde for 10 min, and washed twice with PBS. They were incubated for 3 h in a mixture (1 mg mL^{-1} X-Gal in PBS) containing potassium ferricyanide (5 mM), potassium ferrocyanide (5 mM), and $MgCl_2$ (2 mM). The reaction was stopped by removing the X-Gal reaction solution.

Infectivity assays: PBMC were isolated from buffy coats of healthy humans as described above. Cells were incubated with HIV-1 LAI (0.3 ng) or HIV-1 BaL (0.3 ng) for 2 h at 37 °C in 2% FCS medium in the presence of various amounts of peptides. Infected cells were then washed twice with FCS-free medium and cultured in the presence of peptides in 10% FCS. Every three days, the supernatant was harvested and tested for p24 (Innotest HIV Antigen mAB, Innogenetics).

Peptide stability in the presence of human serum: To determine the stability of C34 and its analogue peptides in human sera, each peptide (100 μg) was incubated at 37 °C for various times and tested for the maintenance of its capacity to inhibit syncytium formation and HIV-1 replication as described above.

Water solubility determination: Water solubility of the peptides was determined by amino acid analysis. Peptide (1 mg) was dissolved in ammonium carbonate (10 mM) at pH 8.0. The solution was neutralized with CH_3CO_2H (1 N) to pH 7.0 and then lyophilized. The sample was re-dissolved in H_2O (1 mL) and was incubated for 12 h at 8 °C. The suspension was centrifuged at 5000 rpm (1200 *g*) for 5 min. The supernatant was filtered through a 0.2 μm filter. The concentration of an aliquot of 500 μL was determined by amino acid analysis.

Acknowledgements

This work was supported by SIDACTION, MRT, and the Université Paul Sabatier.

Keywords: C34 · D-amino acids · HIV-1 · inhibitors · peptides

[1] A. G. Dalgleish, P. C. Beverley, P. R. Clapham, D. H. Crawford, M. F. Greaves, R. A. Weiss, *Nature* **1984**, *312*, 763–767.
[2] D. Klatzmann, E. Champagne, S. Chamaret, J. Gruest, D. Guetard, T. Hercend, J. C. Gluckman, L. Montagnier, *Nature* **1984**, *312*, 767–768.
[3] J. P. Moore, *Science* **1997**, *276*, 51–52.
[4] M. Bendjennat, B. Bahbouhi, E. Bahraoui, *Biochemistry* **2001**, *40*, 4800–4810.
[5] D. Einfeld, *Curr. Top. Microbiol. Immunol.* **1996**, *214*, 133–176.
[6] E. Fenouillet, B. Clerget-Raslain, J. C. Gluckman, D. Guetard, L. Montagnier, E. Bahraoui, *J. Exp. Med.* **1989**, *169*, 807–822.
[7] R. W. Doms, J. P. Moore, *J. Cell. Biol.* **2000**, *151*, 9–14.
[8] W. Weissenhorn, A. Dessen, S. C. Harrison, J. J. Skehel, D. C. Wiley, *Nature* **1997**, *387*, 426–430.
[9] T. Muster, R. Guinea, A. Trkola, M. Purtscher, A. Klima, F. Steindl, P. Palese, H. Katinger, *J. Virol.* **1994**, 68, 4031–4034.
[10] P. Chambers, C. R. Pringle, A. J. Easton, *J. Gen. Virol.* **1990**, *71*, 3075–3080.
[11] A. Holguin, E. R. De Arellano, V. Soriano, *AIDS Res. Hum. Retroviruses* **2007**, *23*, 1067–1074.
[12] B. K. Felber, M. Hadzopoulou-Cladaras, C. Cladaras, T. Copeland, G. N. Pavlakis, *Proc. Natl. Acad. Sci. USA* **1989**, *86*, 1495–1499.
[13] Y. Kliger, A. Aharoni, D. Rapaport, P. Jones, R. Blumenthal, Y. Shai, *J. Biol. Chem.* **1997**, *272*, 13496–13505.
[14] M. Pritsker, P. Jones, R. Blumenthal, Y. Shai, *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 7287–7292.
[15] C. H. Chen, T. J. Matthews, C. B. McDanal, D. P. Bolognesi, M. L. Greenberg, *J. Virol.* **1995**, *69*, 3771–3777.
[16] M. K. Lawless, S. Barney, K. I. Guthrie, T. B. Bucy, S. R. Petteway, G. Merutka, *Biochemistry* **1996**, *35*, 13697–13708.
[17] C. T. Wild, D. C. Shugars, T. K. Greenwell, C. B. McDanal, T. J. Matthews, *Proc. Natl. Acad. Sci. USA* **1994**, *91*, 9770–9774.
[18] T. Matthews, M. Salgo, M. Greenberg, J. Chung, R. DeMasi, D. Bolognesi, *Nat. Rev. Drug Discovery* **2004**, *3*, 215–225.
[19] S. Liu, H. Lu, J. Niu, Y. Xu, S. Wu, S. Jiang, *J. Biol. Chem.* **2005**, *280*, 11259–11273.
[20] I. Munoz-Barroso, S. Durell, K. Sakaguchi, E. Appella, R. Blumenthal, *J. Cell Biol.* **1998**, *140*, 315–323.
[21] S. A. Gallo, A. Puri, R. Blumenthal, *Biochemistry* **2001**, *40*, 12231–12236.
[22] Y. He, R. Vassell, M. Zaitseva, N. Nguyen, Z. Yang, Y. Weng, C. D. Weiss, *J. Virol.* **2003**, *77*, 1666–1671.
[23] G. B. Melikyan, R. M. Markosyan, H. Hemmati, M. K. Delmedico, D. M. Lambert, F. S. Cohen, *J. Cell Biol.* **2000**, *151*, 413–423.
[24] L. T. Rimsky, D. C. Shugars, T. J. Matthews, *J. Virol.* **1998**, *72*, 986–993.
[25] L. Xu, A. Pozniak, A. Wildfire, S. A. Stanfield-Oakley, S. M. Mosier, D. Ratcliffe, J. Workman, A. Joall, R. Myers, E. Smit, P. A. Cane, M. L. Greenberg, D. Pillay, *Antimicrob. Agents Chemother.* **2005**, *49*, 1113–1119.
[26] J. J. Eron, R. M. Gulick, J. A. Bartlett, T. Merigan, R. Arduino, J. M. Kilby, B. Yangco, A. Diers, C. Drobnes, R. DeMasi, M. Greenberg, T. Melby, C. Raskino, P. Rusnak, Y. Zhang, R. Spence, G. D. Miralles, *J. Infect. Dis.* **2004**, *189*, 1075–1083.
[27] J. P. Lalezari, N. C. Bellos, K. Sathasivam, G. J. Richmond, C. J. Cohen, R. A. Myers, Jr., D. H. Henry, C. Raskino, T. Melby, H. Murchison, Y. Zhang, R. Spence, M. L. Greenberg, R. A. Demasi, G. D. Miralles, *J. Infect. Dis.* **2005**, *191*, 1155–1163.
[28] J. J. Dwyer, K. L. Wilson, D. K. Davison, S. A. Freel, J. E. Seedorff, S. A. Wring, N. A. Tvermoes, T. J. Matthews, M. L. Greenberg, M. K. Delmedico, *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 12772–12777.
[29] J. J. Dwyer, K. L. Wilson, K. Martin, J. E. Seedorff, A. Hasan, R. J. Medinas, D. K. Davison, M. D. Feese, H. T. Richter, H. Kim, T. J. Matthews, M. K. Delmedico, *Protein Sci.* **2008**, *17*, 633–643.
[30] C. A. Bewley, J. M. Louis, R. Ghirlando, G. M. Clore, *J. Biol. Chem.* **2002**, *277*, 14238–14245.
[31] S. Y. Hong, J. E. Oh, K. H. Lee, *Biochem. Pharmacol.* **1999**, *58*, 1775–1780.
[32] D. Bang, G. I. Makhatadze, V. Tereshko, A. A. Kossiakoff, S. B. Kent, *Angew. Chem.* **2005**, *117*, 3920–3924; *Angew. Chem. Int. Ed.* **2005**, *44*, 3852–3856.
[33] F. I. Valiyaveetil, M. Sekedat, R. Mackinnon, T. W. Muir, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 17045–17049.
[34] B. Anil, B. Song, Y. Tang, D. P. Raleigh, *J. Am. Chem. Soc.* **2004**, *126*, 13194–13195.
[35] B. Anil, R. Craig-Schapiro, D. P. Raleigh, *J. Am. Chem. Soc.* **2006**, *128*, 3144–3145.
[36] D. C. Chan, P. S. Kim, *Cell* **1998**, *93*, 681–684.
[37] D. C. Chan, D. Fass, J. M. Berger, P. S. Kim, *Cell* **1997**, *89*, 263–273.
[38] D. C. Chan, C. T. Chutkowski, P. S. Kim, *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 15613–15617.
[39] J. D. Reeves, F. H. Lee, J. L. Miamidian, C. B. Jabara, M. M. Juntilla, R. W. Doms, *J. Virol.* **2005**, *79*, 4991–4999.
[40] S. K. Sia, P. A. Carr, A. G. Cochran, V. N. Malashkevich, P. S. Kim, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 14664–14669.
[41] J. K. Judice, J. Y. Tom, W. Huang, T. Wrin, J. Vennari, C. J. Petropoulos, R. S. McDowell, *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 13426–13430.

[42] B. S. Jin, J. R. Ryu, K. Ahn, Y. G. Yu, *AIDS Res. Hum. Retroviruses* **2000**, *16*, 1797–1804.
[43] D. M. Eckert, V. N. Malashkevich, L. H. Hong, P. A. Carr, P. S. Kim, *Cell* **1999**, *99*, 103–115.
[44] B. D. Welch, A. P. VanDemark, A. Heroux, C. P. Hill, M. S. Kay, *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 16828–16833.
[45] G. Guichard, S. Muller, M. van Regenmortel, J. P. Briand, P. Mascagni, E. Giralt, *Trends Biotechnol.* **1996**, *14*, 44–45.
[46] M. Chorev, M. Goodman, *Trends Biotechnol.* **1995**, *13*, 438–445.
[47] M. K. Lee, H. K. Kim, T. Y. Lee, K. S. Hahm, K. L. Kim, *Exp. Mol. Med.* **2006**, *38*, 18–26.
[48] R. Huey, G. M. Morris, A. J. Olson, D. S. Goodsell, *J. Comput. Chem.* **2007**, *28*, 1145–1152.
[49] N. J. Greenfield, *Methods Enzymol.* **2004**, *383*, 282–317.
[50] N. Sreerama, R. W. Woody, *Methods Enzymol.* **2004**, *383*, 318–351.
[51] B. J. Smith, *Enzymatic Cleavage of Proteins In Protein Sequencing Protocols*, **2003**, Humana, Totowa, NJ, pp. 49–62.

Received: November 14, 2008
Revised: December 18, 2008
Published online on February 10, 2009

