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Development of Antiviral Fusion Inhibitors: Short Modified Peptides Derived from the Transmembrane Glycoprotein of Feline Immunodeficiency Virus

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Feline immunodeficiency virus (FIV) is a naturally occurring pathogen that causes an AIDS-like syndrome in domestic cats and is a valuable model system by which criteria for antiviral vaccines and drugs development can be tested. The cell-entry step of the lentivirus life cycle is regarded as a promising target for the development of new generation inhibitors. We have previously described potent in vitro anti-FIV activity associated with a synthetic octapeptide, termed C8 (Ac-Trp-Glu-Asp-Trp-Val-Gly-Trp-Ile-NH$_2$), containing the Trp-rich motif of FIV transmembrane glycoprotein, which shares a common structural framework with the corresponding molecule of HIV and appears to play a similar role in cell entry. In this report, in an attempt to develop simpler potential fusion inhibitors to be tested in vivo, we describe further studies focused on synthetic peptide analogues of C8. Since C8 inhibitory activity is dependent upon the Trp motif, we systematically replaced these residues with bulky and/or aromatic natural and unnatural amino acids, in order to develop a rational structure–activity relationship. Furthermore, the amino acids located between the Trp residues, which are not crucial for inhibitory activity, were replaced by simple alkyl spacers of appropriate length. Design, NMR structural analysis, in vitro anti-FIV activity in lymphoid cell cultures, and serum stability of these new analogues are reported. The final results indicate that a simpler hexapeptide (Ac-Nal2-Ape-Nal2-Ape-Nal2-Ile-NH$_2$; Nal2 = 3-naphthalen-2-yl-L-alanine, Ape = 5-aminopentanoic acid), almost entirely made up of unnatural amino acid residues, has markedly increased enzymatic stability, while maintaining strong antiviral potency in vitro.

Introduction

Despite the success of current antiretroviral therapies in reducing the morbidity and mortality associated with HIV infection, there is still a tremendous effort to develop new agents that are active against resistant HIV strains.[1] The cell entry step of the HIV-1 life cycle is regarded as a promising target for the development of new generation inhibitors,[2] and in fact enfuvirtide, a fusion peptide inhibitor also known as T-20,[3] has been in use in clinical practice since 2004. This 36-residue peptide corresponds to a C-terminal region of the ectodomain of the transmembrane (TM) glycoprotein gp41, a component of the HIV-1 envelope that plays a crucial role in membrane fusion and viral entry.

Feline immunodeficiency virus (FIV) is a naturally occurring pathogen that causes an AIDS-like syndrome in domestic cats and is also a valuable model system in which criteria for antiviral vaccines and drug development can be tested.[4] We have previously described potent in vitro anti-FIV activity associated with synthetic peptides containing the Trp-rich motif of FIV TM glycoprotein,[5] which shares a common structural framework with the corresponding molecule of HIV and appears to play a similar role in cell entry.[6] We focused on the antiviral activity

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of a Trp motif contained in the octapeptide named C8 (Ac-Trp-Glu-Asp-Trp-Val-Gly-Trp-Ile-NH_2), which showed a powerful antiviral effect on all the FIV isolates tested, this activity being dependent on an intact Trp motif. Additional investigations revealed that the structure of peptide C8 is characterized by a well defined β-turn structure resulting in a well defined orientation of the side chains, including the Trp rings. The conformational properties of C8 might explain why it is potently inhibitory in spite of its reduced size.[7]

One of the main concern when testing a peptide, particularly a short one, as a drug candidate is its enzymatic stability, which normally makes its half life in serum too short for therapeutic application. We have, however, recently reported the results of a short-term monotherapy experiment in chronically FIV-infected cats, showing that an enzymatically stable retro-inverso analogue of C8 has—unlike unmodified C8—substantial antiviral activity in vivo.[8] We thus decided to study the structure–activity relationship of C8 in order to obtain information relevant to the rational design of modified peptides, possibly structurally simpler than C8, more suitable for in vivo testing.

Results

Peptide synthesis

All the peptides listed in Table 1 were prepared manually by a solid-phase strategy on TentaGel resin, with Fmoc/*t*Bu chemistry and HBTU/HOBt activation for amide bond formation. After acidic cleavage, performed with TFA containing a suitable scavenger mixture, fully deblocked crude peptides were recovered by ether precipitation and lyophilized. The final products were obtained after extensive HPLC purification on a C18 column, by use of a water/acetonitrile gradient, containing 0.1% TFA, and characterized by analytical RP-HPLC and mass spectrometry.

Table 1. Inhibition of a primary FIV-M2 isolate replication by peptide **C8** and analogues.

No.	Sequence	IC_{50}[a] [μM]
1 (**C8**)	Ac-**Trp**-Glu-Asp-**Trp**-Val-Gly-**Trp**-Ile-NH_2	0.22 ± 0.12
2	Ac-**Trp**-Glu-Asp-**Trp**-Ape-**Trp**-Ile-NH_2	0.97 ± 0.77
3	Ac-**Trp**-Ape-**Trp**-Val-Gly-**Trp**-Ile-NH_2	1.02 ± 0.72
4	Ac-**Trp**-Ape-**Trp**-Ape-**Trp**-Ile-NH_2	0.34 ± 0.11
5	Ac-Lys-**Trp**-Ape-**Trp**-Ape-**Trp**-Ile-NH_2	1.52 ± 0.47
6	Ac-Lys-**Trp**-βAla-**Trp**-βAla-**Trp**-Ile-NH_2	> 50
7	Ac-Lys-**Trp**-Aoc-**Trp**-Aoc-**Trp**-Ile-NH_2	> 50
8	Ac-**Phe**-Glu-Asp-**Phe**-Val-Gly-**Phe**-Ile-NH_2	0.49 ± 0.06
9	Ac-**His**-Glu-Asp-**His**-Val-Gly-**His**-Ile-NH_2	1.82 ± 0.10
10	Ac-**Pal**-Glu-Asp-**Pal**-Val-Gly-**Pal**-Ile-NH_2	0.52 ± 0.23
11	Ac-**Bta**-Glu-Asp-**Bta**-Val-Gly-**Bta**-Ile-NH_2	0.53 ± 0.12
12	Ac-**Bip**-Glu-Asp-**Bip**-Val-Gly-**Bip**-Ile-NH_2	> 50
13	Ac-**Cha**-Glu-Asp-**Cha**-Val-Gly-**Cha**-Ile-NH_2	0.88 ± 0.20
14	Ac-**Nal1**-Glu-Asp-**Nal1**-Val-Gly-**Nal1**-Ile-NH_2	0.24 ± 0.08
15	Ac-**Nal2**-Glu-Asp-**Nal2**-Val-Gly-**Nal2**-Ile-NH_2	0.09 ± 0.08
16	Ac-**Nal2**-Ape-**Nal2**-Ape-**Nal2**-Ile-NH_2	0.34 ± 0.11

[a] Values shown are means ± SDs of the IC_{50} values obtained in two–three independent assays.

Antiviral activity in vitro

Inhibition of FIV replication was tested by the previously described standard procedure.[7] Test peptides were diluted in tissue culture medium to final concentrations ranging from 0.0005 to 50 μM and assayed for virus inhibition against ten 50% tissue culture infectious doses ($TCID_{50}$s) of the fresh FIV-M2 isolate, with lymphoid MBM cells as substrate and p25 antigen quantification as an end point. The final results, reported in Table 1, are expressed as 50% inhibitory concentrations (IC_{50}s). The reference peptide C8 (**1** in Table 1) shows an IC_{50} of 0.22 μM. In a first series of analogues (compounds **2**–**7**), we explored the possibility of replacing the two dipeptide units separating the essential Trp residues with simple alkyl spacers of variable length. We initially used 5-aminopentanoic acid (Ape), formally containing the same number of atoms along the peptide backbone as the unmodified dipeptide unit. Ape was used to replace the C-terminal dipeptide (Val-Gly) and also the N-terminal one (Glu-Asp), either singularly (compounds **2** and **3**, respectively) or together (compound **4**). All three analogues maintain inhibitory activity, but interestingly, while the peptides with a single amino acid replacement each show a fourfold decrease in activity, the analogue **4** with two replacements maintains almost the same inhibitory activity as the native peptide (IC_{50} = 0.34 μM). This compound was further modified by N-terminal elongation with a Lys residue, the amino acid naturally occurring in the corresponding position in the sequence of the FIV TM glycoprotein. This modification was introduced in an attempt to increase the hydrophilicity of the modified peptide, in which two hydrophilic residues (Glu and Asp) of the native peptide had been removed. However, the lysyl analogue (entry no. **5** in Table 1) showed a sixfold decrease in inhibitory activity. We subsequently evaluated the role of the length of the alkyl spacer in the inhibitory activity, replacing Ape by shorter or longer spacers: 3-aminopropionic acid (or β-alanine; βAla) and 8-aminooctanoic acid (Aoc), respectively. Interestingly, the resulting compounds (**6** and **7**) were both devoid of significant inhibitory activity.

The second series of peptides (**8**–**15**) was designed in order to investigate the role of the Trp residues, which had previously been shown to be critical for the inhibitory activity of C8. Accordingly, these three residues were replaced with several bulky and hydrophobic residues, both natural and unnatural (Scheme 1).

The results reported in Table 1 indicate that the three Trp residues can be replaced, provided that a certain bulk and hydrophobicity are maintained. In fact, the inhibitory activity is lost only in the case of 3-biphenyl-4-yl-L-alanine (Bip, peptide **12**), the side chain topology of which is clearly different from that of Trp, and is strongly reduced only in the case of His (**9**), a much smaller and more polar amino acid then Trp. On the other hand, replacement of Trp residues with Phe (**8**), 3-pyridin-2-yl-L-alanine (Pal, **10**), 3-benzothiophen-2-yl-L-alanine (Bta, **11**), and the nonaromatic 3-cyclohexyl-L-alanine (Cha, **13**) brings about moderate reductions in activity (two- to fourfold). Finally, the use of the two naphthylalanine isomers (3-naphthalen-1-yl-L-alanine, Nal1; 3-naphthalen-2-yl-L-alanine, Nal2) yield-

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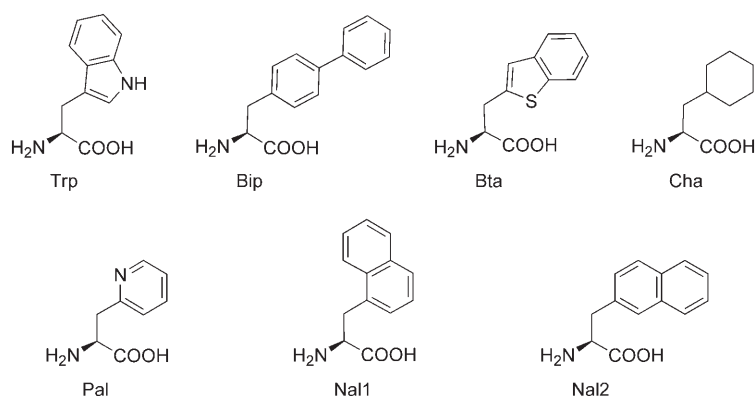

Scheme 1. Structures of unnatural amino acid residues. Bip = 3-biphenyl-4-yl-L-alanine, Bta = 3-benzothiophen-2-yl-L-alanine, Cha = 3-cyclohexyl-L-alanine, Pal = 3-pyridin-2-yl-L-alanine, Nal1 = 3-naphthalen-1-yl-L-alanine, Nal2 = 3-naphthalen-2-yl-L-alanine.

ed two analogues in which inhibitory activity is maintained (Nal1, peptide **14**) or even increased (Nal2, peptide **15**, IC_{50} = 0.09 μM) relative to that of C8. All the compounds had no detectable toxicity up to 50 μM for cultured cells.

Finally, compound **16** was obtained, combining the most favorable modification of each series: the Ape spacer in place of the two central dipeptide units and Nal2 in place of Trp. Interestingly, the resulting compound, formally a hexapeptide and almost completely made up of unnatural amino acid residues, maintains the same potent antiviral activity as the unmodified octapeptide C8 (IC_{50} = 0.34 μM).

Serum stability

Since the final goal of this study was the rational development of an analogue of peptide C8 more suitable for in vivo testing, a key issue to be investigated was the serum stability of compound **16**. Accordingly, compound **16** was compared with C8 for inhibitory activity and resistance to enzymatic degradation in normal cat serum (NCS). The results in Table 2 show that the inhibitory activity of peptide C8 is reduced over 20-fold in the presence of 50% NCS, in comparison with a less than fivefold decrease for **16**.

To assess their enzymatic stabilities, the extents of degradation of peptides **16** and C8 were estimated by measuring the residual peptide concentration by LC-ESI-MS after incubation in NCS. The test peptides were diluted to 17.7 μg mL^{-1} in NCS and incubated at 37 °C. At selected times, the protein degradation was stopped by MeCN addition (serum protein precipitation) and the residual intact peptide in the supernatants, collected after centrifugation, was estimated by quantitative LC-ESI-MS with use of internal standards. We initially observed that the protein precipitation procedure, based on MeCN addition to NCS, enables quantitative recovery of the added peptides, thus showing that neither peptide **16** nor C8 bind to serum proteins. These data rule out differences between **16** and C8 in solubility, aggregation, and nonspecific serum-binding properties. After incubation at 37 °C for 24 h in NCS, the residual concentration of C8 was only 30% of the initial one, while compound **16** proved to be more stable, showing a residual concentration of 55%.

Table 2. Effect of NCS on the inhibition of FIV replication by peptides **C8** and **16**.

Peptide	IC_{50}[a] [μM] Medium	NCS
C8	0.22 ± 0.12	4.60 ± 1.86
16	0.34 ± 0.11	1.64 ± 1.09

[a] Values shown are means ± SDs of the IC_{50} values obtained in two independent assays.

Conformational studies

A detailed NMR investigation was undertaken in order to correlate the biological activity profiles of C8 analogues with their structural features. Accordingly, we recorded 1D and 2D ^{1}H NMR spectra of some biologically significant C8 derivatives. In particular, from the first group of analogues (**2**–**7**), we decided to analyze the active compound **4** and the inactive analogue **6**, while from the second group (**8**–**15**) we chose the active compound **14** and the inactive compound **12**.

Several sets of NMR conditions were explored for the chosen C8 derivatives, spectra being run in water, DMSO, and DMSO/water (80:20, *v*/*v*). Because of the structure-stabilizing properties of DMSO/water mixtures[9] the full NMR study of the selected peptides was performed in DMSO/water (80:20, *v*/*v*) at 290 K. Chemical shift assignments of proton spectra were achieved by standard systematic application of DQF-COSY,[10] TOCSY,[11] and NOESY[12] experiments, with use of the SPARKY[13] software package according to Wüthrich's procedure.[14] Figure 1 summarizes the most significant NOE connectivities for each analogue. Inspection of the diagrams in Figure 1 shows the presence of nontrivial, sequential NOE effects suggestive of

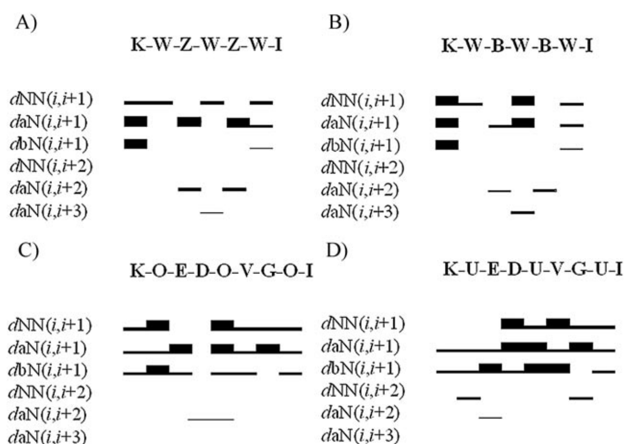

Figure 1. Short- and medium-range NOEs relating to the NOESY spectra of compounds a) **4**, b) **6**, c) **12**, and d) **14** recorded at 290 K in DMSO/water (80:20, *v*/*v*; Bruker 600 MHz). Z = Ape, B = βAla, C = Bip, U = Nal1.

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the presence of folded ordered conformers. In particular, sequential NH–NH effects are diffusively observable together with crucial α-NH ($i,i+2$) or α-NH($i,i+3$) relationships diagnostic of turn-folded structures.

Three-dimensional structures were calculated from the NOE data by simulated annealing in torsion angle space by use of the DYANA software package[15] and refined by minimization with the SANDER module of the AMBER 5.0 software package.[16] According to the presence of the diagnostic medium-range NOEs (Figure 1), the calculated models show folded structures stabilized by H bonds. In particular, H bonds involving the NH of Trp3 and the CO of Ape4 are observable in many of the NMR calculated structures of compound **4**. On the other hand, H-bonds between the NH of Trp4 and the CO of Ile7 are detectable in a significant number of the calculated structures relating to compound **6** (Figure 2). The H bonds stabilizing structures **12** and **14** are basically present in their central and N-terminal regions (Figure 3). In particular, many of the conformers relating to the analogue **14** are characterized by H bonds between the CO of Glu2 and the NH of Nal4, whereas in compound **12** the presence of H bonds involving the CO of Bip1 and the NH of Bip4 or alternatively the NH of Bip1 and the CO of Asp3 is systematically observable.

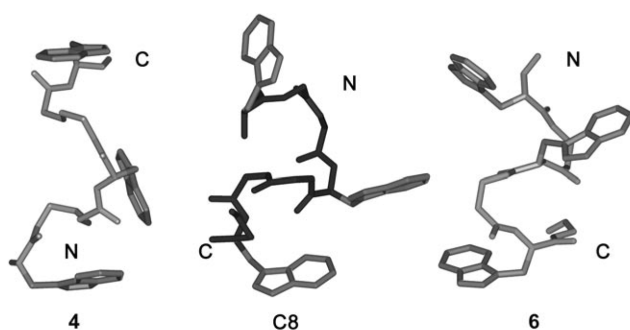

Figure 2. NMR structures of compounds **4** and **6** in comparison with that of the reference compound C8.[7] The models define the most representative structures of those obtained by DYANA calculation. The N and C termini of each peptide are labeled, to specify the antiparallel alignment.

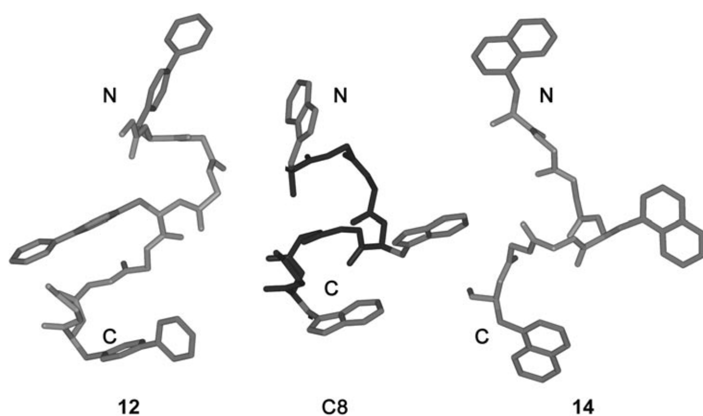

Figure 3. NMR structures of compounds **12** and **14** in comparison with that of the reference compound C8.[7] The models define the most representative structures of those obtained by DYANA calculation. The N and C termini of each peptide are labeled.

Discussion

We have previously described the potent anti-lentiviral activity of C8, a short peptide encompassing the Trp-rich motif of the FIV TM glycoprotein, and have shown by alanine scan that the peculiar Trp motif of this peptide is critical for inhibitory activity.[7] Since the limited bioavailability of peptides, particularly of short ones, is a well known drawback hampering further in vivo exploitation of this molecule, we undertook this study with the goal of the rational development of a modified peptidomimetic analogue more suitable for in vivo testing.

The previously reported structure–activity relationship data for C8 clearly indicate the possibility of replacing the two dipeptide units Glu-Asp and Val-Gly with simpler spacing moieties. Accordingly, we successfully replaced both these dipeptides with an alkyl spacer of optimal length (i.e., Ape) formally containing the same number of backbone atoms as the unmodified dipeptide unit (compound no. **4** in Table 1). In spite of the greater flexibility of the alkyl spacers in relation to the dipeptide units, the maintenance of the optimal distance between the three Trp residues proved to be crucial for inhibitory activity, as clearly shown by the lack of activity of the analogues bearing a shorter (**6**) or a longer spacer (**7**). This result is in line with our previously reported structural studies, showing a peculiar topological arrangement of the Trp side chains.[7]

The biological data are further supported by the NMR results. Figure 2 compares the calculated structures of compounds **4** and **6** with that previously reported for the reference compound, C8. As mentioned above, turn structures involving the C-terminal residues are present in all three peptides. A comparative analysis of the NMR models of the active compounds **4** and C8 interestingly shows that these two structures can be superimposed, provided that the backbones are in an antiparallel alignment. This is possibly due to the high symmetry of compound **4**, since this is the only way to have the two consecutive Trp rings exposed toward a common surface. In contrast, comparison of the inactive compound **6** with C8 shows no steric possibility to overlap these structures in an arrangement yielding a common orientation of the Trp rings. This might be due to the short distance between the Trp residues obtained with the shorter spacer (βAla), which induces a backbone arrangement minimizing the interactions between the Trp rings, resulting in orientations in opposite directions.

The biological activities of the analogues bearing Trp replacements (compounds **8**–**15**) are also quite interesting because they suggest that the interaction between C8 and its molecular target has specific requirements not only from a structural viewpoint, as shown previously,[7] but also in terms of topology of the interacting side chains: that is, the key Trp residues. In fact, in spite of a certain degree of tolerance to substitution, a high level of inhibitory activity is maintained only in a few analogues, and particularly those bearing the two Nal isomers as Trp replacements (compounds **14** and **15**).

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This result indicates that hydrophobicity and, most importantly, steric bulk are critical determinants of the inhibitory activities of these peptides.

The importance of the aromatic side chains' bulk and orientation as a biological activity discriminating factor becomes clearer on comparison of the NMR structures of compounds **12** and **14** with that of the reference peptide C8, shown in Figure 3. Once again, comparison between the active analogue **14** and C8 shows conformity between these structures, since compound **14** has a turn structure in which the orientation of the aromatic rings in C8 is conserved. On the other hand, the inactive compound **12** adopts a turn structure that does not allow the aromatic rings to be exposed on the same face of the molecule. Moreover, the lack of activity of peptide **12** could be dependent on the presence of the biphenyl-alanine residues. These amino acids, characterized by a nonplanar relative orientation of the two phenyl rings, confer steric and electronic properties significantly different from those of C8 on this compound.

Taken together, these data clearly indicate that, besides bulk and hydrophobicity, the interaction of the inhibitory peptide with its molecular target depends upon shape and orientation of the key side chains. This sensitivity to very subtle changes in topological structure (1- to 2-naphthyl) suggests that the peptides interact with a specific binding site. Similar observations were reported by Chan et al.[17] for the HIV inhibitory peptide C34, in which the Trp motif was found to interact with a specific prominent cavity in the N-terminal portion of gp41. Moreover, the crucial role of orientation in the interaction between peptide inhibitors such as T-20 and their molecular target(s) has been recently underlined by Peisajovich et al.[18] These indications support the hypothesis that C8 is a suitable template for the rational development of a drug candidate.

Another important issue relating to the drug-like properties of C8 analogues is enzymatic stability. Several reports indicate that incorporation of non-α amino acids (e.g., Ape)[19] and unnatural residues (e.g., Nal)[20] increases stability against proteases. Accordingly, we found that compound **16** is less prone to degradation than C8 in normal cat serum and so appears to be more suitable for in vivo testing.

In conclusion, these findings show that an inhibitory peptide designed to mimic the Trp motif of FIV can undergo modifications that markedly increase enzymatic stability without affecting the antiviral potency. The degrees of enzymatic stability achieved by some of the modifications described should permit testing for in vivo antiviral activity.

Experimental Section

Starting materials: N^{α}-Fmoc-protected natural and nonnatural amino acids, HBTU, HOBt, and PyBOP were purchased from Fluka–Sigma–Aldrich (Milan, Italy) or from Advanced ChemTech–FKV (Sorisole, Italy). TentaGel S RAM resin was purchased from Fluka–Sigma–Aldrich. Peptide synthesis solvents and reagents and CH_3CN for HPLC were reagent grade and were purchased from Riedel–Sigma–Aldrich (Milan, Italy) and used without further purification.

Peptide synthesis: Synthesis was performed manually, in a stepwise fashion, by the solid-phase Fmoc/*t*Bu method. For peptides **1**–**7**, protected amino acids were added stepwise to TentaGel S RAM resin (capacity: 0.24 $mmol\,g^{-1}$); each coupling reaction was accomplished through the use of a fourfold amino acid molar excess with activation with an equimolar excess of HBTU/HOBt in the presence of DIEA or NMM (sixfold excess) for 40 min. Peptides **8**–**16** were synthesized by use of a fourfold molar excess of protected amino acids and PyBOP (0.5 M), without any previous activation. The peptide resin was subsequently washed with DMF (2×) and DCM (2×), and the completeness of the coupling reaction was checked by the Kaiser test. The N^{α}-Fmoc protecting groups were removed after each coupling cycle by treatment of the protected peptide resin with piperidine in DMF (30%) for 10 min. After deprotection of the last N-terminal residue, the peptide was acetylated with acetic anhydride (20-fold excess) and DIEA (40-fold excess) in DMF (2 mL). Finally, the peptide was released from the resin with TFA/H_2O/TIPS (90:5:5, *v*/*v*/*v*, 90 min). The resin was removed by filtration and the crude peptide was recovered by precipitation with cold ethyl ether to give a white powder. Finally, the crude peptide was dissolved in CH_3COOH/H_2O (1:50, *v*/*v*) and lyophilized. Peptides containing Trp residues (**1**–**7**) were maintained in solution for two days at room temperature before lyophilization, in order to remove the Boc group from the indolic nitrogen completely. Analytical RP-HPLC was carried out with a Beckmann–Coulter system, consisting of a 125S Solvent System Gold model fitted with a 166 Detector System Gold. Preparative RP-HPLC was carried out on a Waters (Millipore) system consisting of a 600E System Controller, a 486 Tunable Absorbance Detector, and a 746 Date Module Integrator. Analytical runs were performed on a Phenomenex–Jupiter C18 column (250×4.6 mm, 5 μm, 300 Å pore size), with a linear gradient (5–71% in 22 min) of CH_3CN in water containing 0.1% TFA, at a flow rate of 1 $mL\,min^{-1}$. UV detection: 210 nm. Peptide purification was performed on a semipreparative Vydac C18 218TP (10×250 mm, 10 μm, 300 Å pore size) column with a linear gradient starting from 20% of CH_3CN (0.15 $\%\,min^{-1}$min) in water containing 0.1% TFA, at a flow rate of 4 $mL\,min^{-1}$. UV detection: 210 nm. The final product was obtained by lyophilization of the appropriate fractions after removal of the CH_3CN by rotary evaporation. Analytical RP-HPLC indicated $>97\%$ purity. Molecular masses were determined on a Finnigan LCQ-Deca ion trap instrument fitted with an electrospray source (San Jose, CA). Analytical data are reported as Supporting Information.

Inhibitory activity: Virus inhibition assay was performed in 96-well flat-bottomed microplates against ten 50% Tissue Culture Infectious Doses ($TCID_{50}$s) of FIV, with MBM cells as substrate and testing of the supernatant fluids for p25 at day 8 post-inoculation. Virus was appropriately diluted and mixed with an equal volume of the test peptides diluted from 0.005 to 50 μM (final concentrations) or solvent alone. The mixtures were then immediately inoculated into MBM cells and washed out after 5 h at 37 °C. Supernatants were tested for p25 antigen by ELISA as described previously.[7] Peptide inhibition of virus growth was calculated by use of the formula (mean p25 concentration in wells inoculated with FIV + peptides/mean p25 concentration in wells inoculated with FIV alone)×100. Fifty percent inhibitory concentrations (IC_{50}s) were calculated by use of the predicted exponential growth function in Microsoft Excel. Mean IC_{50}s ± standard deviations (SDs) were calculated with all replicates. All experiments were repeated at least twice.

Serum stability: Quantitation of residual peptide **16** after incubation in serum was carried out by mass spectrometry, with use of

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the peptide Ac-Nal2-Ape-Nal2-Ape-Nal2-Val-NH_2 as internal standard for **16** and Ac-Trp-Glu-Asp-Trp-Ile-Gly-Trp-Ile-NH_2 for C8. The test peptides, dissolved at 100 $\mu g\,mL^{-1}$ in a TFA solution (0.1%) of H_2O/CH_3CN (1:1) and stored at $-20\,^{\circ}C$ until use, were diluted to 17.7 $\mu g\,mL^{-1}$ in NCS and incubated at 37 °C. At selected times, aliquots (100 µL) were taken and mixed with CH_3CN (300 µL) to stop any ongoing degradation, as well as with the internal standard (4 $\mu g\,mL^{-1}$). All the experiments were performed in duplicate. After centrifugation at 10000 g for 10 min, the supernatants were injected onto a C18 column (Vydac 218MS51 C18 Mass Spec column; 5 µm, 250 Å, 1.0 mm) and eluted with a gradient mobile phase of 0.1% aq. HCOOH and 0.1% HCOOH in CH_3CN (20 to 100% over 20 min) at a flow rate of 50 $\mu L\,min^{-1}$ into the LCQ Advantage mass spectrometer (ThermoFinnigan, San Jose, CA) with electrospray interface. For peptide **16** positive single-charged ions were monitored in the full scan mode (900–1000 *m/z*), while for C8 negative single-charged ions were monitored in the 1000–1300 *m/z* mass ranges. The calibration range of the internal standard curves in NCS was 0.5 to 200 $\mu g\,mL^{-1}$.

NMR spectrometry: Samples for NMR were prepared by dissolving the appropriate amount of each C8 analogue to obtain a solution in DMSO/H_2O (1 mM, 0.5 mL, 80:20, *v/v*). The water part contained ^2H_2O (20 µL). NMR spectra were recorded on a Bruker DRX 600 spectrometer fitted with a cryogenic probe. 1D NMR spectra were recorded in the Fourier mode with quadrature detection. The water signal was suppressed by low-power selective irradiation in the homogated mode. DQF-COSY, TOCSY, and NOESY experiments were run in the phase-sensitive mode with use of quadrature detection in ω1 by time-proportional phase incrementation of the initial pulse. Data block sizes comprised 2048 addresses in t2 and 512 equidistant t1 values. Before Fourier transformation, the time domain data matrices were multiplied by shifted $\sin^2$ functions in both dimensions. A mixing time of 70 ms was used for the TOCSY experiments. NOESY experiments were run at 290 K with mixing times in the range of 250–350 ms. The qualitative and quantitative analyses of DQF-COSY, TOCSY, and NOESY spectra were performed with the aid of the SPARKY interactive program package.

NMR structure calculation: The 3D models of compounds **4, 6, 12**, and **14** were calculated by use of simulated annealing procedures with the aid of the DYANA software package. The distribution of NOE-derived restraints as intraresidue range, short range, and medium range is reported in Figure 1 for each peptide. The calculations for the compounds containing the modified amino acids (Nal, Bip, Ape, βAla) were carried out with an appropriate expansion of the DYANA amino acid library. All structures were energy-minimized with the SANDER module of the AMBER 5 program, by the steepest descent method for 1000 steps and the conjugate gradient method for 4000 steps. A nonbonded cut-off of 12 Å and a distance-dependent dielectric term ($e = 4 \times r$) were used. The minimization protocol included three steps in which NOE-derived distances were used as constraints with force constants of 1000, 100, and 10 $kcal\,mol^{-1}$Å, respectively. The final pdb file was subjected to a PROCHECK protocol for validation of the geometry. The analysis of the 3D structures was performed on a SGI indigo II workstation with use of INSIGHTII software.

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