Design of a Gag Pentapeptide Analogue that Binds Human Cyclophilin A More Efficiently than the Entire Capsid Protein: New Insights for the Development of Novel Anti-HIV-1 Drugs†

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Received June 17, 1999

Cyclophilin A (hCyp-18), a ubiquitous cytoplasmic peptidyl-prolyl cis/trans isomerase (PPIase), orchestrates HIV-1 core packaging. hCyp-18, incorporated into the virion, enables core uncoating and RNA release and consequently plays a critical role in the viral replication process. hCyp-18 specifically interacts with a single exposed loop of the Gag polyprotein capsid domain via a network of nine hydrogen bonds which mainly implicates a 7-mer fragment of the loop. As previously reported, the corresponding linear heptapeptide Ac-Val-His-Ala-Gly-Pro-Ile-Ala-NH_2 **(2)** binds to hCyp-18 with a low affinity ($IC_{50} = 850 \pm 220$ μM) but a potentially useful selectivity for hCyp-18 relative to hFKBP-12, another abundant PPIase. On the basis of X-ray structures of Gag fragments:hCyp-18 complexes, we generated a series of modified peptides in order to probe the determinants of the interaction and hence to select a peptidic ligand displaying a higher affinity than the capsid domain of Gag. We synthesized a series of heptapeptides to test the energetic contribution of amino acids besides the Gly-Pro moiety. In particular the importance of the histidine residue for the interaction was underscored. We also investigated the influence of N- and C-terminal modifications. Hexapeptides containing either deaminovaline (Dav) in place of the N-terminal valine or substitution of the C-terminal alanine amide with a benzylamide group displayed increased affinities. Combination of both modifications gave the most potent competitor Dav-His-Ala-Gly-Pro-Ile-NHBn **(28)** which has a higher affinity for hCyp-18 ($K_d = 3 \pm 0.5$ μM) than the entire capsid protein ($K_d = 16 \pm 4$ μM) and a very low affinity for hFKBP-12. Some of our results strongly suggest that the title compound is not a substrate of hCyp-18 and interacts preferentially in the trans conformation.

Introduction

Immunophilins are ubiquitous proteins which exhibit peptidyl-prolyl cis/trans isomerase (PPIase) activity.[1−4] They play a critical role in protein synthesis by accelerating a rate-limiting step during protein folding. PPIases are therefore implicated in many biological processes related to protein folding and in several diseases.[5] PPIases are divided into several families, among which cyclophilins and FK506-binding proteins (FKBP) are the most abundant and best-characterized. Cyclophilin A (hCyp-18), the archetypal member of the cyclophilin subfamily, is an 18-kDa cytoplasmic protein. It is the main receptor for the immunosuppressant cyclic undecapeptide cyclosporin A (CsA) which is currently employed for the prevention of graft rejection.[6,7]

In 1994, Luban and co-workers demonstrated that hCyp-18 is integrated inside the viral core of HIV-1, the pathogenic agent of AIDS, and is essential for the release of viral RNA and hence for the multiplication of the virus.[8−11] More precisely, hCyp-18 interacts specifically with the capsid domain of the dimerized Gag polyprotein[12,13] and promotes the assembly of the viral core.[14] In subsequent steps of the viral maturation, the polyprotein is cleaved into three distinct proteins: matrix protein (MA), capsid protein (CA), and nucleocapsid protein (NC), which remain stacked together. About 250 hCyp-18 molecules are packaged into each virion in a ratio of 1 hCyp-18 to 10 CA.[15] The exact function of hCyp-18 in the HIV-1 viral cycle is still controversial. Some results suggest that it destabilizes the viral core and enables the virus uncoating,[9,16] but this theory has been recently questioned.[17−19] Disruption of hCyp-18 incorporation by mutation in Gag[10] or maturation in the presence of CsA[8,20] during virion budding causes a quantitative reduction in viral infectivity, though hCyp-18 is not strictly required for virion assembly.[21] Neither circulating nor cytosolic hCyp-18 can rescue hCyp-18-deficient virions,[9] which are no longer infectious.

Numerous anti-HIV drugs have been designed to target viral enzymes. However error-prone reverse transcription and high rates of retroviral recombination as well as the decreasing adhesion of many patients to long-term treatments have led to the emergence of viruses resistant to combined therapies.[22] Consequently, the definition of novel pharmacological targets is critical. Host proteins implicated in the viral replication cycle, such as hCyp-18, are not prone to genetic instability and offer new potential therapeutic applications.[15]

† This paper is dedicated to the memory of Prof. Michel Gaudry.

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10.1021/jm9903139 CCC: $19.00
Published on Web 04/18/2000

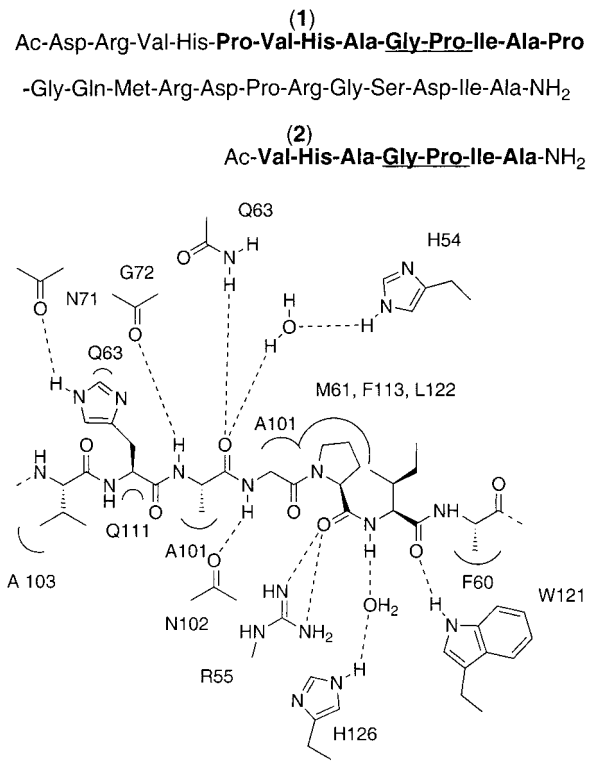


Figure 1. Sequence of peptides **1** and **2**. Schematic representation of the hCyp-18:Gag[86−92] interactions.[13] van der Waals contacts involving Pro85 and Pro93 have been omitted for clarity. The H-bond between Ile91 and W121 is very weak in the CA:Gag interaction.

Cyclosporin derivatives devoid of immunosuppressive activity are interesting inhibitors since CsA is a selective high-affinity ligand of hCyp-18.[20,23−27] However, synthesis of series of cyclic undecapetides is generally time-consuming[28,29] and conceptually difficult because the global conformation of the molecule is strongly influenced by side-chain modifications.[30] Moreover, cyclosporin-based treatments cause chronic renal and hepatic toxicities,[31] facilitate apoptosis,[32] and induce cancer progression.[33]

Several nonisomerizable *cis*-aminoacyl-proline mimetics[34−36] have been described. Even though interesting affinities were observed, no data regarding the hCyp-18/hFKBP-12 selectivity was reported. A potential transition-state analogue ketoamide-containing peptidomimetic also displayed micromolar affinities for both hCyp-18 and hFKBP-12 and hence no selectivity.[37,38]

Another possible strategy is the design of small linear peptides or pseudopeptides mimicking a fragment of HIV-1 proteins which interacts with hCyp-18. Even though a 20-mer peptide derived from the V3 loop of the GP120 protein tightly binds hCyp-18, the interaction is not selective since it also interacts with CypB (hCyp-20) and hFKBP-12 with submicromolar affinities.[39] Very recently Gehring and co-workers have reported that a 15-mer peptide derived from the capsid C-terminal domain is a high-affinity ligand of both hCyp-18 and hCyp-20 and is able to inhibit PPIase activity.[45] However, no data regarding the selectivity for hCyp-18 versus hFKBP-12 were reported. Recently, Fisher and co-workers have shown that a 25-mer peptide (Figure 1, peptide **1**) derived from CA[81−105] interacts with hCyp-18 with both moderate affinity and selectivity.[41]

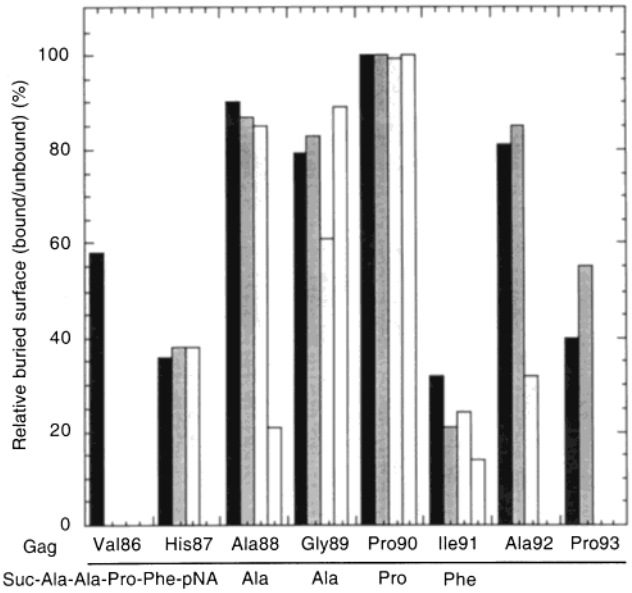


Figure 2. Residues burying in hCyp-18-bound peptides. Relative buried surfaces of complexed fragments of Gag[13,43,45] were calculated using the program AREAIMOL v. 3.5 (CCP4 suite, Peter Brick, Imperial College). N- and C-termini have been omitted when they are not inserted into a peptidic structure. The *cis*-A-P-containing substrate tetrapeptide has been lined up for comparison: solid bars, CA[1−145]; gray-shaded bars, CA[8−105]; open bars, CA[87−92]; Suc-Ala-Ala-Pro-Phe-pNA.

A smaller peptide corresponding to the CA[86−92] fragment (Figure 2, peptide **2**) possesses a lower affinity but a better selectivity for hCyp-18 toward hFKBP-12.[42] Structures of hCyp-18 as well as different hCyp-18:ligand complexes have been resolved by NMR and X-ray crystallography. In particular, structures of complexes between hCyp-18 and either the N-terminal fragment of CA[1−151][13] or the 25-mer peptide[43] have been determined, and the minimal binding domain has been delineated. hCyp-18 interacts with a reduced portion of an exposed loop of the capsid domain via a set of 2 water-mediated and 7 direct hydrogen bonds, 18 van der Waals contacts, and no ionic interactions (Figure 1). Except His2 imidazole N-H, the H-bonding network exclusively concerns the Ala3-Ile6 peptidic backbone. Here we denote hCyp-18-derived residues with the one-letter code, while the three-letter code will be employed for the ligands. For clarity, the numbering of the amino acids from the Gag protein will be conserved, while Gag-derived peptides will be numbered starting from Val86.

In CA fragment/peptide complexes, the peptide interacts with the enzyme's hydrophobic pocket[44] in an unprecedented *trans*-Gly-Pro conformation because of stereochemical restraints.[13] This was confirmed by Vajdos et al. who resolved the structure of hCyp-18 complexed with the hexapeptide [87−92] HAGPIA.[45] This suggests that the interaction is basically different from those observed with other Xaa-Pro peptidic substrates[46−48] and results in full occupancy of the hydrophobic pocket (Figure 2). As expected, the hydrophobic pocket of hCyp-18 delimited by M61, F113, L122, and A101 closely encompasses the pyrrolidine ring of Pro90 (Figure 2). This explains the marked specificity of cyclophilins for the proline residue or proline isosteres, in contrast with the relative tolerance observed for the FKBPs.[2] The low affinity of the 25-mer lead

peptide (IC_{50} = 180 μM)[41] suggests that the loop conformation is also important for the stability of the complex.[43]

The design of small linear peptides or peptidomimetics derived from the Gag loop should lead to the development of novel anti-HIV drugs capable of irreversibly blocking the viral cycle. Such linear peptides should not display immunosuppressive properties since they do not possess a calcineurin interaction moiety.[49] We therefore focused on the heptamer **2** as a lead peptide because it combines a moderate affinity with an interesting selectivity for hCyp-18. We report here the synthesis, biochemical evaluation, and structure–activity relationship of short peptidic and pseudopeptidic ligands of hCyp-18 potentially capable of disrupting the Gag:hCyp-18 interaction. We evaluated the importance of each residue and asymmetric center of the lead peptide for the interaction with Gag. Then we generated series of modified peptides in order to select a short hCyp-18 ligand displaying a micromolar affinity comparable with the affinity of the capsid protein.[50]

Chemistry

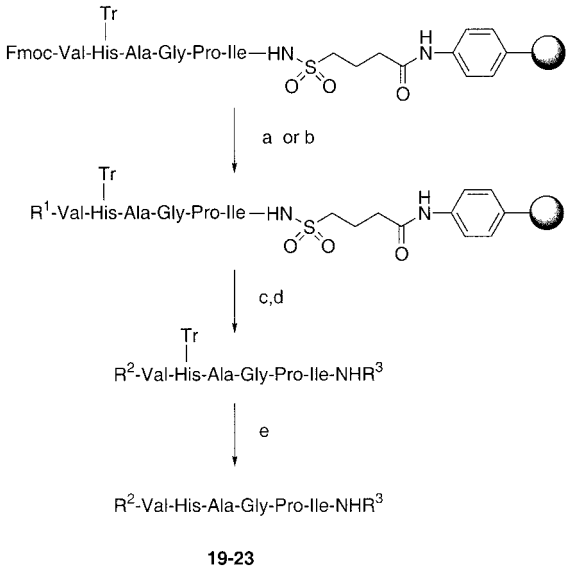
Using the standard Fmoc strategy, C-terminal free amide peptides **2**–**17** were synthesized by polymer-supported peptide synthesis onto a Rink-amide resin. α-Disubstituted amino acids were coupled using the PyBOP procedure. The coupling of phenylglycine with PyBOP resulted in a complete epimerization of this residue. Both peptides containing either a L- or D-phenyglycinyl moiety could be separated by RP-HPLC and thus were tested separately. Their absolute configurations were not determined.

The preparation of C-terminal-substituted amide hexapeptides was not obvious though several strategies using classical or activatable resins have been reported.[51–54] We first chose to employ the 4-sulfamylbutyryl AM resin (Kenner's "safety catch" linkers) recently described by Ellman and co-workers.[55,56] This resin enables the synthesis of peptides using either Boc or Fmoc standard procedures (except for the first step which requires PyBOP double-coupling) because it is reasonably stable to trifluoroacetic acid and piperidine. Subsequent activation of the sulfamide with iodoacetonitrile, splitting, and displacement using nucleophilic primary and secondary amines led to the substituted amide peptides **19**–**23** (Scheme 1). In the case of free amine peptide **14**, the intermediary Fmoc-deprotected peptide was transiently capped with diterbutyl dicarbonate in order to avoid a preferential reaction of the amine with iodoacetonitrile during the activation step. However, the nucleophile-directed displacement was only successful with nucleophilic and unhindered amines. In particular, no reaction took place with dibenzylamine. To our knowledge, this is the first time that functionalized hexapeptides have been synthesized using the 4-sulfamylbutyryl AM resin.[57]

Modified pentapeptides were obtained by solution peptide synthesis using a mixed Boc/Fmoc strategy (Scheme 2). Boc-deaminohistidine (Boc-Dah) was obtained in two steps from urocanic acid. Fmoc deprotection using diisopropylamine and capping afforded the target compounds in good overall yields (Scheme 2).

Synthesis of compounds **44**–**46** was performed by standard solution peptide synthesis as depicted in Scheme 3. The intermediary Fmoc-peptide methyl esters **41**–**43** were converted into the corresponding benzylamides by treatment with benzylamine and trimethylaluminum in refluxing dichloromethane as previously reported.[58]

Scheme 1. Synthesis of C-Terminal N-Substituted Amide Peptides **19**–**23** Using 4-Sulfamylbutyryl Resin[a]



[a] (a) For R^2 = isovaleryl, acetyl, or succinyl: 20% piperidine in NMP, isovaleryl chloride or acetyl chloride or succinic anhydride, DIPEA in NMP; for R^2 = H: (b) 20% piperidine in NMP, Boc_2O, DIPEA in NMP; (c) iodoacetonitrile, DIPEA in dry DMF, overnight; (d) R^3-NH_2 in THF, 4 h; (e) TFA/TIPS/DCM (5/5/90), 30 min.

Peptide **52** was synthesized by a different strategy using Fmoc-DL-isoserine (DL-Ise) as an aminopyruvate (Apy) precursor. The free secondary alcohol **51** was oxidized with PDC in dichloromethane.[59] The crude product was deprotected with TFA without intermediary purification, and RP-HPLC purification yielded the target compound **52**.

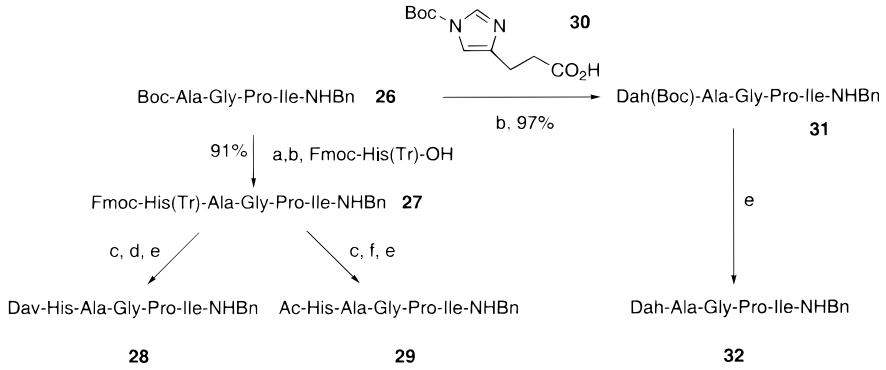
Results and Discussion

Peptides were tested as competitors of hCyp-18 PPIase activity using the uncoupled assay described by Fischer and co-workers.[60] Isomerization of the substrate peptide Suc-Ala-Ala-Pro-Phe-DFA was monitored by UV spectrophotometry at 246 nm at 10 ± 0.1 °C in order to minimize spontaneous interconversion. Analysis of the pseudo-first-order kinetics in the presence of an increasing inhibitor concentration gave the IC_{50} as summarized in Tables 1 and 2.

The IC_{50} for compound **2** (850 ± 220 μM) was in agreement with the previously reported value (710 ± 30 μM) determined under similar experimental conditions.[42] Replacement of His2 with Gln (an uncharged H-bond donor or acceptor with a similar length) caused a significant decrease in affinity (compound **3**), while introduction of ornithine (a charged H-bond donor) resulted in a complete lack of affinity (compound **4**, Table 1). This indicates that the imidazole ring is critical for the affinity.[61]

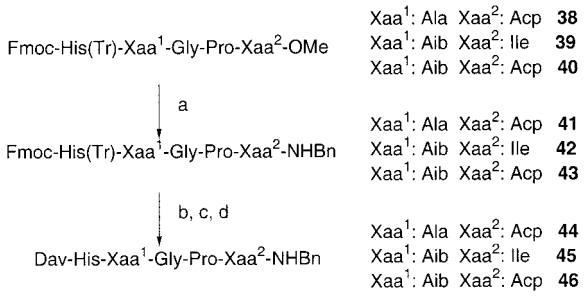
The relatively low affinity of the lead heptapeptide Ac-Val-His-Ala-Gly-Pro-Ile-Ala-NH_2 **(2)** indicates that the loop conformation and dynamics are important factors for the development of Gag antagonists. The environment of Ala3 suggests that this position is well-

Scheme 2. Solution Synthesis of Peptides **28** (Dah-His-Ala-Gly-Pro-Ile-NHBn), **29** (Ac-His-Ala-Gly-Pro-Ile-NHBn), and **32** (Dah-His-Ala-Gly-Pro-Ile-NHBn)[a]



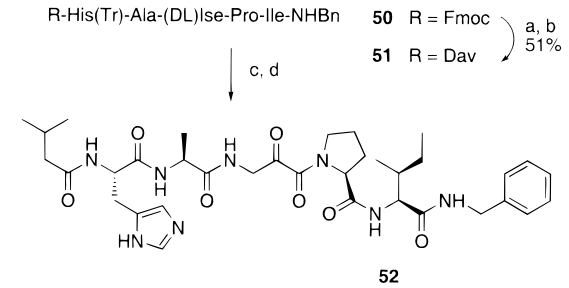
[a] (a) TFA/DCM, 30 min; (b) DCC, HOBT in DCM; (c) 20% diisopropylamine in DMF, 1 h; (d) isovaleryl chloride, DIPEA in DMF; (e) TFA/TIPS/DCM (5/5/90), 30 min; (f) acetyl chloride, DIPEA in DMF.

Scheme 3. Solution Synthesis of Peptides **44** (Dah-His-Ala-Gly-Pro-Acp-NHBn), **45** (Dah-His-Aib-Gly-Pro-Ile-NHBn), and **46** (Dah-His-Aib-Gly-Pro-Acp-NHBn)[a]



[a] (a) Bn-NH_2, $AlMe_3$ in refluxing DCM; (b) 20% diisopropylamine in DMF, 1 h; (c) isovaleryl chloride, DIPEA in DMF; (d) TFA/TIPS/DCM (5/5/90), 30 min.

Scheme 4. Solution Synthesis of Peptide **52**[a]



[a] (a) 20% diisopropylamine in DMF, 1 h; (b) isovaleryl chloride, DIPEA in DMF; (c) PDC, 3 Å molecular sieves in DCM; (d) TFA/water/DCM (5/5/90), 30 min.

suited for the introduction of conformational restraints (Figure 2). This was very recently confirmed by Fischer and co-workers who demonstrated that replacement of Ala3 by its D-isomer does not affect the affinity.[62] Substitution of Ala3 with aminoisobutyric acid (compound **5**), an achiral helix and β-bend inductor, did not modify the activity of the peptide.

The influence of Ile6 cannot be clearly inferred from structural analysis of hCyp-18 complexes. In all complexes the side chain protrudes outside the hCyp-18 recognition site (Figure 2), and in the Gag structure itself it is fully buried.[13] To evaluate the influence of this position on the conformation of Gag loop and on vicinal H-bonds, various homologues were tested. Peptide **6** which has no side chain appears to be a rather poor ligand. The Ile6Val (peptide **7**) and Ile6Gln (peptide **8**) analogues of peptide **2** displayed a weak but significant affinity for hCyp-18. Surprisingly, substitution of Ile6 with L- or D-phenylglycine did not detectably affect the affinity. This suggested that nonchiral α-disubstituted amino acids could be introduced at this position.

Table 1. Percentages of Inhibition at 500 μM and IC_{50} ± Standard Deviation of Peptides **2**−**13**[a]

compd	sequence	% inhib (500 μM)	IC_{50} ± SD (n = 2) (μM)
2	Ac-Val-His-Ala-Gly-Pro-Ile-Ala-NH_2	19	850 ± 220
3	Ac-Val-**Gln**-Ala-Gly-Pro-Ile-Ala-NH_2		2500
4	Ac-Val-**Orn**-Ala-Gly-Pro-Ile-Ala-NH_2		>10000
5	Ac-Val-His-**Aib**-Gly-Pro-Ile-Ala-NH_2	45	760 ± 310
6	Ac-Val-His-Ala-Gly-Pro-**Gly**-Ala-NH_2	28	2800 ± 300
7	Ac-Val-His-Ala-Gly-Pro-**Val**-Ala-NH_2	31	1500 ± 300
8	Ac-Val-His-Ala-Gly-Pro-**Gln**-Ala-NH_2	15	1300 ± 200
9	Ac-Val-His-Ala-Gly-Pro-LD-**Phg**-Ala-NH_2	43[b]	820 ± 290[b]
10		37[c]	970 ± 400[c]
11	Ac-Val-His-Ala-Gly-Pro-**Aib**-Ala-NH_2		>10000
12	Ac-Val-His-Ala-Gly-Pro-**Apc**-Ala-NH_2		>10000
13	Ac-Val-His-Ala-Gly-Pro-**Acp**-Ala-NH_2	52	680 ± 180

[a] Isomerization of the substrate peptide Suc-Ala-Ala-Pro-Phe-DFA was monitored at 246 nm in the presence of increasing concentrations of compounds **2**−**13** in DMSO (2-min preincubation at 10 °C), in a pH 7.8 35 mM Hepes buffer at 10 ± 0.5 °C using 16 nM human hCyp-18. [b] First diastereomer eluted by RP-HPLC. [c] Second diastereomer.

Table 2. Percentages of Inhibition at 500 μM and IC_{50} ± Standard Deviation for Peptides **14**−**23**, **28**, **29**, **32**, and **44**−**45**[a]

compd	sequence	% inhib (500 μM)	IC_{50} ± SD (n = 2) (μM)
14	**Val-His**-Ala-Gly-Pro-Ile-Ala-**NH_2**	11	2100 ± 300
15	Suc-Val-His-Ala-Gly-Pro-Ile-Ala-**NH_2**	13	1000 ± 800
16	Dav-His-Ala-Gly-Pro-Ile-Ala-**NH_2**	76	165 ± 70
17	Dah-Ala-Gly-Pro-Ile-Ala-**NH_2**	20	2600 ± 500
18	His-Ala-Gly-Pro-Ile-Ala	15	2000 ± 200
19	Val-His-Ala-Gly-Pro-Ile-**NH-CH_2-Ph**	59	260 ± 130
20	Ac-Val-His-Ala-Gly-Pro-Ile-**NH-CH_2-Ph**	88	115 ± 45
21	Ac-Val-His-Ala-Gly-Pro-Ile-**NH-CH(Ph)$_2$**	38	1500 ± 900
22	Ac-Val-His-Ala-Gly-Pro-Ile-**NH-CH_2CH_2-Ph**	55	325 ± 145
23	Ac-Val-His-Ala-Gly-Pro-Ile-**NH-CH(Ph)$_2$**	54	365 ± 155
28	Dav-His-Ala-Gly-Pro-Ile-**NH-CH_2-Ph**	99	6 ± 2[b]
29	Ac-His-Ala-Gly-Pro-Ile-**NH-CH_2-Ph**	55	280 ± 100
32	Dah-Ala-Gly-Pro-Ile-**NH-CH_2-Ph**		>10000
44	Dav-His-**Ala**-Gly-Pro-**Acp-NH-CH_2-Ph**	57	90 ± 45
45	Dav-His-**Aib**-Gly-Pro-**Ile-NH-CH_2-Ph**		>10000
46	Dav-His-**Aib**-Gly-Pro-**Acp-NH-CH_2-Ph**	50	500 ± 130
	Suc-Ala-Ala-Pro-Phe-pNA	38	850 ± 300
	CsA		0.016 ± 0.004

[a] Isomerization of the substrate peptide Suc-Ala-Ala-Pro-Phe-DFA was monitored at 246 nm in the presence of increasing concentrations of inhibitors in DMSO (2-min preincubation at 10 °C) in a pH 7.8 35 mM Hepes buffer at 10 ± 0.5 °C using 16 nM human hCyp-18. [b] n = 5.

A similar result was obtained with 2-aminocyclopentanecarboxylate (Acp) (compound **13**) but not with 2-aminoisobutyrate (Aib) or 4-aminopiperidine-4-carboxylate (Apc), a hydrophilic homologue of Acp, which completely lacked affinity. This latter result was rather unexpected because the charged and midly hydrophilic piperidine group should favor the interaction with hCyp-18 by protruding toward the solvent. These data indicate that Ile6 may be conveniently replaced with the nonchiral residue Acp and that hCyp-18 does not tolerate the presence of a charged side chain in the C-terminal part of the interacting peptide.

We also anticipated that the introduction of a positive charge at the N-terminus should decrease the affinity whereas hydrophobic capping was expected to improve the binding. This was confirmed with compounds **14**−**16** (Table 2). The best result was obtained with peptide **16** ($IC_{50} = 165 \pm 70$ μM) which lacks the N-terminal part of the backbone but contains a valine side-chain equivalent (Dav). On the other hand, further reduction of the peptide size resulted in a dramatic increase of IC_{50} (compound **17**).

The close contact of Ala7 methyl with the F60 aromatic ring and the relatively hydrophobic environment surrounding this residue hint that a hydrophobic peptide C-terminus and more precisely an aromatic moiety should improve the affinity. The replacement of Ala7 with various benzyl- and phenethylamides led to a significant improvement of the inhibition. This may be explained by a head-to-tail or a $\pi-\pi$ interaction of the cycle either with F60 aromatic ring (which interacts with the Ala92 in the wild-type sequence) or with W121 (which interacts with Pro90). However, increase in chain length or ring number did not seem to improve the interaction (see Table 2, compounds **19**−**23**).

Finally, peptide **28**, which combines the isovaleryl moiety (Dav) with the benzylamide terminus, displayed a valuable improvement in potency, implying an additive effect of N- and C-terminal modifications since an IC_{50} of 6 ± 2 μM was observed. This result was further confirmed by measuring the K_d of **28** for hCyp-18. This was achieved by fluorescence titration of hCyp-18 W121 in the presence of increasing concentrations of peptide.[23] Compound **28** ($K_d = 3 \pm 0.5$ μM) has an affinity higher than that of the capsid protein ($K_d = 16 \pm 4$ μM)[50] and equivalent to that of the CA[81−117] peptide ($K_i = 8.3 \pm 0.8$ μM).[40] In the same conditions, the substrate peptide Suc-Ala-Ala-Pro-Phe-pNA and CsA displayed respective K_d values of 135 ± 20 and 0.48 ± 0.08 μM.

The shortening of the isovaleryl into an acetyl at the N-terminus of the peptide (compound **29**) resulted in a 45-fold decrease in affinity, whereas substitution of the Dav-His fragment with a deaminohistidine (Dah) (peptide **32**) caused a complete loss of affinity. These data, as well as the results obtained with peptides **3**, **4**, and **17**, suggest that His2 is a critical residue for the affinity not only through the interaction of the ring itself but also through the general conformation of the side chain.

Peptides **44**−**46** corresponded to the combination of each substitution investigated with compounds **5**, **13,** and **28**. The Ile6Acp substitution caused a 15-fold decrease in efficiency (compound **44**), while the double change Ala3Aib/Ile6Acp resulted in a 100-fold increase in IC_{50} (compound **46**). This combination of negative effects may be easily explained by the multiplication of conformational restrictions, which no longer allow the correct interaction. Unexpectedly, compound **45,** which presents only the Ala3Aib modification, does not interact with hCyp-18. Compared with findings obtained for compounds **2** and **5**, this result suggests that the interaction of the N- and C-termini-modified peptides is rather different from that of the lead peptide. It could reflect not only a multiplication of van der Waals contacts but also a closer fitting of the peptide inside the hCyp-18 binding site.

To improve the affinity of Gag-derived pentapeptides, we combined the high-affinity sequence with a potential transition-state analogue of the PPIase activity. Even though the implication of PPIase activity in the Gag: hCyp-18 interaction is not clearly established, recent results suggest that the enzyme-catalyzed isomerization of the Gag Gly89-Pro90 moiety could be critical for the polyprotein processing and the destabilization of the viral core.[40] The mechanism of isomerization itself is still unclear.[63,64] Most recent results suggest that it involves a deconjugated transition-state with a quaternarization of nitrogen and a free-bond rotation of the "ketoamine" intermediate.[65] This was supported by the generation of catalytic antibodies possessing PPIase activity, using ketoamide-containing haptens. As expected, such peptides inhibited both hCyp-18 and hFKBP-12 in the micromolar range.[37,38] Moreover, the amide surrogate has been found in very high-affinity FKBP ligands such as FK506, rapamycin, ascomycin, and other noncyclic synthetic analogues.[66] We inserted an aminopyruvyl-proline (Apy), a putative deconjugated Gly-Pro mimetic, inside the modified pentapeptide **28**. Unfortunately, compound **52** is not an inhibitor of hCyp-18, and no interaction could be detected at concentrations up to 200 μM. Two explanations might be proposed. First, the highly specific interaction does not allow nonisosteric backbone modifications. Indeed the unusual *trans*-Gly-Pro conformation suggests that the interaction is basically different from those observed with other Xaa-Pro peptidic substrates[46−48] and results in full occupancy of the hydrophobic pocket (Figure 2). This implies that the Gly-Pro moiety should be conserved or replaced with isosteres since it is an important element for both affinity and selectivity. Second, the PPIase activity may not be strictly required for the hCyp-18:Gag interaction, and a transition-state analogue of the reaction intermediate cannot be used as a lead to design a very potent inhibitor.

Compound **28** was also tested with recombinant hFKBP-12 using the uncoupled assay. The catalyzed isomerization of Suc-Ala-Leu-Pro-Phe-pNA[69] was monitored at 330 nm. Though IC_{50} values for hCyp-18 and hFKBP-12 cannot be strictly compared, the relatively high IC_{50} (3300 ± 500 μM) against hFKBP-12 shows that peptide **28** is a selective ligand of hCyp-18. In fact, Gly-Pro-containing peptides are weak substrates of hFKBP-12 which preferentially binds the Leu-Pro sequence.[63] In the same conditions, the IC_{50} of ascomycin, a potent selective hFKBP-12 inhibitor, was 8 ± 2 nM.

Finally, we further characterized the mode of action of compound **28** on cyclophilin. Particularly, we wanted to discriminate whether it acts as a competing substrate or an inhibitor. We first analyzed the influence of the

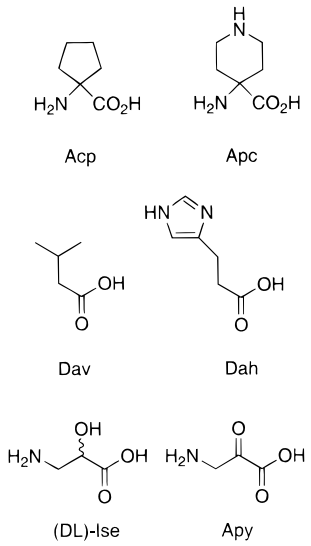


Figure 3. Structures of unusual amino acids: 4-aminopiperidine-4-carboxylate (Apc), 2-aminocyclopentanecarboxylate (Acp), side-chain mimics deaminovaline (Dav) and deaminohistidine (dihydrourocanic acid) (Dah), aminopyruvate precursor isoserine (Ise), and keto-amine surrogate aminopyruvate (Apy).

initial cis/trans ratio on the inhibition to determine the most efficient binding conformation. Indeed, the cis:trans equilibrium of Xaa-Pro-containing peptides is strongly influenced by lithium chloride, and the cis/trans ratio is significantly higher in LiCl/TFE than in buffers.[69] Compound **28** in 0.47 M LiCl/TFE was added to hCyp-18, and the PPIase activity was monitored as usual after varying duration of preincubation. The test was first performed at 4 °C in order to minimize the noncatalyzed isomerization.

The inhibition curves (Figure 4) indicate that compound **28** is less efficient in the presence of LiCl without preincubation. This was further confirmed by mesuring the IC_{50} value with LiCl since a dramatic change was observed ($IC_{50} = 28 \pm 7$ μM). These results are in agreement with an NMR study of the 25-mer peptide[41] and strongly suggest that compound **28** interacts preferentially in the trans conformation.

A time-dependent inhibition was observed (Figure 4, solid circles) whereas no time-dependent variation of the inhibition was detected in the absence of lithium chloride (Figure 4, empty circles). This result is consistent with a slow isomerization process from the LiCl-modified cis/trans ratio. The observed duration of interconversion is rather consistent with a noncatalyzed isomerization. Though the determination of the precise mode of action of this peptide needs additional experiments, we propose that peptide **28** is not a substrate of hCyp-18 and probably acts as a specific inhibitor in the trans conformation.

Conclusion

Cyclophilin A plays an important role in HIV-1 viral infections. As a consequence, disruption of the hCyp-18:CA interaction seems to be a promising new objective in the design of anti-AIDS drugs, which could be used in synergy with "cocktail therapies". In this paper, we report the synthesis and biochemical evaluation of short peptides derived from the capsid sequence. Among other modifications, substitution of the valine residue with a

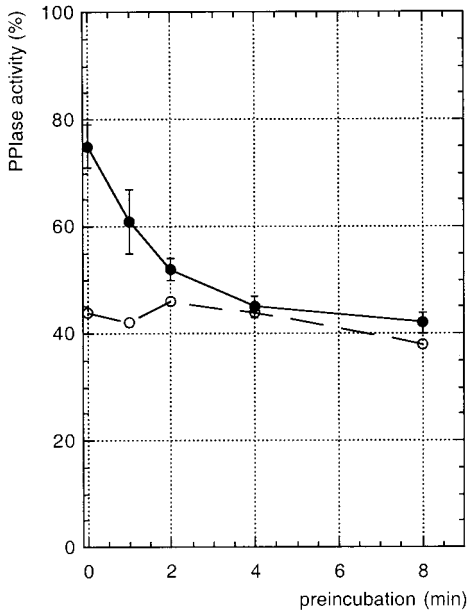


Figure 4. Time-dependent inhibition of hCyp-18 as a function of preincubation duration in a 35 mM Hepes buffer (pH 7.8) using 16 nM human hCyp-18. The PPIase activity was monitored at 246 nm using the standard spectrophotometric uncoupled assay at 4 ± 0.1 °C. Compound **28**, solubilized in either TFE or 0.47 M LiCl in TFE, was added to 16 nM hCyp-18 in a 35 mM Hepes buffer (pH 7.8) at 4 ± 0.1 °C: ○, solution of **28** in TFE; ●, solution of **28** in 0.47 M LiCl in TFE; final concentrations, **28** = 10 μM and LiCl = 9.4 mM.

deaminovaline moiety as well as increase of the C-terminus hydrophobicity with a benzylamide led to a significant improvement in affinity. Combination of both modifications provided pentapeptide **28**, the most potent and selective hCyp-18 ligand in this series, which displayed micromolar affinity. Several residues could be substituted with nonchiral surrogates inside the original sequence without significantly altering the affinity. However, these results were not confirmed with the modified peptides **44**−**46** whose sequence could not be changed without loss of affinity. Our results strongly suggest that the title compound preferentially binds hCyp-18 in a *trans*-Gly-Pro conformation and is not a substrate of hCyp-18. On the other hand, peptide **28** has a greater selectivity for hCyp-18 than for hFKBP-12. Therefore, the modified pentapeptide,which exhibits a slightly higher affinity than the capsid protein and a high selectivity toward hCyp-18, may be an attractive lead for the design of peptidomimetics usable as novel anti-AIDS agents.

Experimental Section

Abbreviations: Ac, acetyl; Acp, 2-aminocyclopentanecarboxylate; Apc, 4-aminopiperidine-4-carboxylate; Apy, aminopyruvate; CA, capsid protein; CsA, cyclosporin; hCyp-18, human cyclophilin A (18 kDa); hCyp-20, mature human cyclophilin B (20 kDa); Dah, deaminohistidine (dihydrourocanic acid); Dav, deaminovaline (isovaleric acid); DCC, dicyclohexylcarbodiimide; DCM, dichloromethane; DFA, 2,4-difluoroaniline; DIPEA, diisopropylethylamine; DMSO, dimethyl sulfoxide; FKBP, FK506-binding protein; HOBT, *N*-hydroxybenzotriazole; NMP, *N*-methylpyrrolidone; pNA, *p*-nitroaniline; PPIase, cis/trans peptidyl-prolyl isomerase; PyBOP, (benzotriazol-1-yloxy)-trispyrrolidinophosphonium hexafluorophosphate; Suc, succinyl; TFA, trifluoroacetic acid; TFE, trifluoroethanol; TIPS, triisopropylsilane.

Materials and Methods. All reagents employed were of analytical grade and were purchased from Aldrich Chemical Co. and Sigma. Amino acids and coupling reagents were obtained from Novabiochem and Bachem. Peptides were purchased from Bachem. THF was distilled before use from sodium benzophenone. All other solvents were of analytical grade and were used without further purification. Flash chromatography was performed on 40−60 μm (230−400 mesh) Merck silica gel. NMR δ and J values are given in ppm and Hz, respectively. High-pressure liquid chromatography was performed on a Waters 625 LC HPLC system coupled to a Waters 991 photodiode array detector. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AVANCE 250 NMR spectrometer. Electrospray mass spectra (Atheris Laboratories, Geneva, Switzerland) were recorded on a Micromass Platform II (Micromass, Altrincham, U.K.). Mass combustion analyses were carried out by the Service de Microanalyse of the ICSN (Gif-sur-Yvette, France). Kinetic assays were performed using a Kontron Uvikon 930 spectrophotometer. Fluorescence titrations were measured with a JASCO FP-750 spectrofluorometer.

Solid-Phase Peptide Synthesis Using the Rink-Amide Resin. Peptides synthesis were performed using Rink-amide resins (0.47−0.53 mmol/g) and DCC/HOBT as coupling reagents in NMP and a 4-fold excess of amino acids in the presence of DIPEA following the standard procedure. α-Substituted amino acids were coupled with PyBOP/HOBT in the presence of DIPEA in NMP. Fmoc deprotections were done by double treatment with a mixture of 20% piperidine in NMP. Final deprotection and cleavage from the polymer was achieved with a mixture of TFA:TIPS:water (90:5:5). After removal of the solvent, the crude product dissolved in glacial acetic acid was purified by RP-HPLC (C_{18} Bondasorb semipreparative column) using a linear gradient 100% TFA (0.1%) in water to 100% acetonitrile in 30 min (flow rate: 4 mL/min). The elution was monitored at 220−250 nm. The peptides were freeze-dried and their purity was checked by analytical RP-HPLC (C_{18} Bondasorb analytical column) using the same gradient.

Compound, formula, calcd MW, ESMS (*m*/*z*), RP-HPLC retention time (min): **2**, $C_{32}H_{52}N_{10}O_8$, 704.8, 704.8, 9.07; **3**, $C_{31}H_{53}N_9O_9$, 695.8, 695.8, 9.52; **4**, $C_{31}H_{55}N_9O_8$, 681.8, 681.9, 8.46; **5**, $C_{33}H_{54}N_{10}O_8$, 718.9, 719.0, 10.16; **6**, $C_{28}H_{44}N_{10}O_8$, 648.7, 648.7, 7.76; **7**, $C_{31}H_{50}N_{10}O_8$, 690.8, 690.8, 8.58; **8**, $C_{31}H_{49}N_{11}O_9$, 719.8, 719.9, 7.83; **9**, $C_{34}H_{48}N_{10}O_8$, 724.8, 724.9, 9.43; **10**, $C_{34}H_{48}N_{10}O_8$, 724.8, 724.9, 10.31; **11**, $C_{30}H_{48}N_{10}O_8$, 676.8, 676.5, 8.87; **12**, $C_{32}H_{51}N_{11}O_8$, 717.8, 717.9, 7.31; **13**, $C_{32}H_{50}N_{10}O_8$, 702.8, 702.9, 9.26; **14**, $C_{30}H_{50}N_{10}O_7$, 662.8, 662.8, 7.53; **15**, $C_{34}H_{54}N_{10}O_{10}$, 762.9, 762.9, 9.20; **16**, $C_{30}H_{50}N_{10}O_6$, 646.8, 647.7, 9.86; **17**, 548.6, 548.7, 7.98.

Automated Solid-Phase Peptide Synthesis Using the Wang Resin. The synthesis of peptide **18** was performed on an Applied Biosystem automated peptide synthesizer using the Fmoc/DCC−HOBT strategy and a Fmoc-Ala-Wang resin (0.6 mmol/g) with a 10-fold excess of reagents. One-step cleavage and deprotection and purification was performed as reported above.

Compound, formula, calcd MW, ESMS (*m*/*z*), RP-HPLC retention time (min): **18**, $C_{26}H_{40}N_8O_7$, 564.7, 564.6, 7.40.

Solid-Phase Peptide Synthesis Using the 4-Sulfamylbutyryl AM Resin. The 4-sulfamylbutyryl AM resin (1.1 mmol/g) was swollen in DCM for 1 h. The resin was then washed with DMF and treated with a Fmoc-amino acid (4.4 mmol), PyBOP (4.4 mmol) and DIPEA (8.8 mmol) for 18 h. The coupling was repeated in the same conditions. After washing with DMF, DCM and hexane, the resin was isolated and the loading was determined by spectrophotometric assay. After standard Fmoc deprotection reaction, the amino acids were coupled by classical manual SPPS Fmoc strategy. After last Fmoc cleavage, the resin-bound peptide was treated with di-*tert*-butyl dicarbonate (4 equiv) and DIPEA (8 equiv) in DMF. The resin was then thoroughly washed with DMF and activated overnight by treament with iodoacetonitrile (4.4 mmol) and DIPEA (1.1 equiv) in DMF under argon. After washing with DMF and THF, the resin was splitted into five equal portions and treated with benzylamine, dibenzylamine, phenylethylamine or diphenylethylamine (4 equiv) in THF for 4 h. The resin was washed twice with THF and removed by filtration. The combined filtrates were evaporated. Deprotection was achieved by treatment with a mixture of TFA:water:TIPS (90:5:5) for 1.5 h. After removal of the solvent under reduced pressure, the crude product was dissolved in acetic acid and purified by RP-HPLC as reported above.

Compound, formula, calcd MW, ESMS (*m*/*z*), RP-HPLC retention time (min): **19**, $C_{34}H_{51}N_9O_6$, 681.8, 681.6, 11.49; **20**, $C_{36}H_{53}N_9O_7$, 723.9, 723.9, 11.90; **21**, $C_{42}H_{57}N_9O_7$, 800.0, 799.9, 15.99; **22**, $C_{37}H_{55}N_9O_7$, 737.9, 737.9, 13.47; **23**, $C_{43}H_{59}N_9O_7$, 814.0, 813.9, 15.62.

Solution Peptide Synthesis: General Procedures. Coupling procedure for Boc-amino acids: Boc-protected peptides were synthesized by the standard solution peptide synthesis method using DCC (1.1 equiv), HOBT (1 equiv) and DIPEA (2 equiv after neutralization) in DCM (5 mL/mmol) for 16 h. After quenching of the reaction by addition of several drops of acetic acid, the precipitate was eliminated by filtration and the solvent was evaporated in vacuo. The product was purified by silica gel flash chromatography (eluent: chloroform:methanol, 95:5) without prior workup. **Boc-deprotection:** The Boc-protected peptide was treated with a mixture of TFA:DCM (50:50) for 30 min at 0 °C. The solvent was removed in vacuo and the product was washed twice with toluene. **Coupling procedure for Fmoc-His(-Tr)-OH:** The deprotected peptide was treated with DIPEA (6.5 equiv after neutralization), Fmoc-His(Tr)-OH (1 equiv), HOBT (1 equiv) and DCC (1 equiv) in DCM (10 mL/mmol) for 80 h at room temperature. After quenching of the reaction by addition of several drops of acetic acid, the precipitate was eliminated by filtration and the solvent was evaporated in vacuo. The product was purified by silica gel flash chromatography (eluent: chloroform:methanol, 95:5) without prior workup. **Fmoc-deprotection:** Compound **27** (210 mg, 0.2 mmol) was treated with a mixture of diisopropylamine:DMF (20:80) (20 mL) for 1 h at room temperature. **Capping:** The peptide was capped using acyl chloride (1 equiv) and DIPEA (2 equiv) in DMF, overnight at room temperature. **Histidine side-chain deprotection and peptide purification:** The trityl group was removed as follows: the peptide was dissolved in a mixture of TFA (4.5 mL), DCM (5 mL) and TIPS (0.5 mL) and the mixture was stirred 1 h at room temperature. After removal of the solvent, the crude product was washed twice with toluene. The residue dissolved in glacial acetic acid was purified by RP-HPLC (C_{18} Bondasorb semipreparative column) using a linear gradient 100% TFA (0.1%) in water to 100% acetonitrile in 30 min (flow rate: 4 mL/min). The elution was monitored at 220−250 nm. The peptides were freeze-dried and their purity was checked by analytical RP-HPLC (C_{18} Bondasorb analytical column) using the same gradient.

Dav-His-Ala-Gly-Pro-Ile-NHBn, 28. Purification by RP-HPLC as reported above gave **28**: t_R = 13.17 min; ^{1}H NMR (CD_3OD) δ 8.78 (d, minor, J = 1.4) + 8.74 (d, major, J = 1.4) (1H), 7.32 (d, J = 1.4) + 7.29 (m) (6H), 4.69 (t, J = 7.0, 1H), 4.53−4.42 (m, 2H), 4.37 (s, 2H),4.26 (d, minor, J = 8.6) + 4.16 (d, major, J = 8.2) (1H), 7.06 (d, major, J = 3.0) + 4.0, (bd) (2H), 3.73−3.54 (m, 2H), 3.14 (m, 2H), 2.2−1.8 (m, 8H), 1.54 (m, 1H), 1.37 (d, J = 7.1, 3H), 1.14 (m, 1H), 0.93−0.84 (m, 12H); ^{13}C NMR (CD_3OD) δ 176.3, 176.2, 175.3, 174.4, 172.5, 170.4, 140.7, 136.0, 131.4, 130.4, 129.4, 129.1, 119.7, 62.4, 60.5, 54.1, 46.7, 44.8, 43.7, 38.8, 31.5, 29.3, 28.2, 27.0, 26.6, 23.5, 18.9, 16.8, 12.2; calcd MW 667.8; ESMS 666.7. Anal. ($C_{34}H_{52}N_8O_6$) C, H, N.

Ac-His-Ala-Gly-Pro-Ile-NHBn, 29. Fmoc-deprotected peptide **27** (0.1 mmol) was treated with acetyl chloride (71 μL, 1 mmol) and DIPEA (175 μL, 1 mmol) in DMF (10 mL) for 1 h at room temperature. Further treatment with the TFA:water:TIPS cocktail and purification by RP-HPLC (C_{18} Vydac semipreparative column) as described above yielded **27**: t_R = 12.63 min; ^{1}H NMR (CD_3OD) δ 8.78 (d, minor, J = 1.3) + 8.74 (d, J = 1.4) (1H), 7.42 (d, J = 1.4) + 7.41−7.21 (m) (6H), 4.67 (dd, J = 6.0, J' = 6.9, 1H), 4.52 (m, 1H), 4.43 (AB, J_{AB} = 7.2, δ_A =

4.47, δ_B = 4.39), 4.37 (s, 2H), 4.22 (d, minor, J = 8.5) + 4.15 (d, major, J = 8.2) (1H), 4.07 (d, major, J = 1.8) + 3.99 (d, minor, J = 2.2), 3.75−3.5 (m, 2H), 3.25−3.05 (m, 2H), 2.3−1.8 (m) + 1.99 (s, minor) + 1.97 (s, major) (8H), 1.7−1.45 (m, 1H), 1.38 (d, J = 7.2, 3H), 1.35−1.05 (m, 1H), 0.95−0.84 (m, 6H); ^{13}C NMR (CD_3OD) δ (major rotamer) 176.3, 175.4, 174.4, 174.0, 172.5, 170.4, 140.7, 136.0, 131.2, 130.4, 129.4, 129.1, 119.7, 62.4, 60.5, 54.3, 44.8, 43.7, 38.7, 31.6, 29.3, 27.0, 26.6, 23.3, 18.8, 16.8, 12.2; calcd MW 624.7; ESMS 624.4. Anal. ($C_{31}H_{45}N_8O_6$) C, H, N.

Dah-Ala-Gly-Pro-Ile-NHBn, 32. Compound **29** (134 mg, 0.20 mmol) was treated with a mixture of TFA:water:TIPS (90:5:5) for 45 min at room temperature. Removal of the solvent and purification by RP-HPLC (C_{18} Vydac semipreparative column) using a linear gradient 100% TFA (0.1%) in water to 100% acetonitrile in 30 min (flow rate: 4 mL/min) yielded **32**: t_R = 10.97 min; ^{1}H NMR (CD_3OD) δ 8.75 (d, minor, J = 1.4) + 8.72 (d, J = 1.4) (1H), 7.33−7.18 (m, 6H), 4.48 (m, 1H), 4.41−4.36 (m, 2H), 4.25−4.13 (m, 1H), 4.03 (AB, J_{AB} = 16.9, δ_A = 4.09, δ_B = 3.96), 3.62 (m, 2H), 3.0 (dt, J = 1.4, J' = 7.2, 2H), 2.61 (bt, 2H), 2.25−1.75 (m, 5H), 1.57 (m, 1H), 1.34 (d, J = 7.2, 3H), 1.3−1.05 (m, 1H), 0.96−0.84 (m, 6H); ^{13}C NMR (CD_3OD) δ 176.2 (major) + 176.1 (minor), 175.3 (major) + 174.9 (minor), 174.4 (major) + 174.3 (minor), 170.4 (major) + 170.2 (minor), 163.6 (minor) + 163 (major), 140.7, 135.5, 135.3, 135.2, 130.3 (129.4 (2 peaks), 129.1 (minor) + 129.0 (major), 118.0 (2 peaks), 62.4 (major) + 61.7 (minor), 60.5 (2 peaks), 44.8, 43.7 (major) + 43.4 (minor), 38.7 (major) + 38.4 (minor), 35.8, 34.2, 31.5, 27.0 (minor) + 26.9 (major), 26.6, 24.3 (minor) + 22.1 (major), 18.7, 16.8, 12.2 (major) + 12.0 (minor); calcd MW 568.7; ESMS 567.8. Anal. ($C_{29}H_{43}N_7O_5$) C, H, N.

General Procedure for Conversion of Fmoc-peptide Methyl Esters into Fmoc-peptide Benzylamides. Benzylamine (2.2 equiv) was added dropwise to a solution of 2 M trimethylaluminum in hexane (2.2 equiv) in dry DCM (10 mL/mmol). The solution was stirred for 30 min at room temperature. The Fmoc-peptide methyl ester in solution in dry DCM (20 mL/mmol) was added and the mixture was stirred 30 min at room temperature then refluxed overnight. After cooling of the reaction medium, the reaction was quenched with 30 mL of 2 M hydrogen chloride at 0 °C. The aqeous layer was extracted twice with DCM. The combined organic layers were washed with brine and then dried over sodium sulfate.

Dav-His-Ala-Gly-Pro-Acp-NHBn, 44. Purification by RP-HPLC as reported above gave **45**: t_R = 13.24 min; ^{1}H NMR (CD_3OD) δ 8.79 (d, 1H, J = 1.33), 8.50 (s, 1H), 8.29 (d, 1H, J = 6.4), 8.18 (t, 1H, J = 6.6), 8.05 (bs, 1H), 7.32−7.19 (m, 8H), 4.69 (t, 1H, J = 6.3), 4.47−4.20 (m, 4H), 4.00 (AB, 2H, JAB = 17.0, δ_A= 4.07, δ_B=3.94), 3.62 (t, 2H, J = 6.3), 3.16 (dd, 2H, J = 6.27, J = 17.6), 2.30−1.77 (m, 15H), 1.32 (b, 3H, J = 7.2), 0.92−0.88 (m, 6H); ^{13}C NMR (CD_3OD) δ 176.5, 175.5, 175.4, 174.7, 171.5, 169.3, 140.5, 135.2, 130.5, 129.4 (minor) + 129.3 (major), 128.4 (minor) + 128.2 (major), 127.9, 118.8, 68.4, 61.9, 53.1, 50.2, 45.9, 44.1, 42.8, 39.2, 37.0, 30.3, 27.3, 26.1, 25.6, 25.4, 25.1, 22.7, 18.2; calcd MW 664.8; ESMS 664.6. Anal. ($C_{34}H_{48}N_8O_6$) C, H, N.

Dav-His-Aib-Gly-Pro-Ile-NHBn, 45. Purification by RP-HPLC as reported above gave **44**: t_R = 14.52 min; ^{1}H NMR (CD_3OD) δ 8.74 (d, 1H, J = 1.41), 8.27 (s, 1H), 7.34 (d, 1H, J = 1.01), 7.32−7.19 (m, 5H), 5.49 (s, 1H), 4.63−4.39 (m, 2H), 4.38 (d, 2H, J = 2.6), 4.19−3.90 (m, 3H), 3.65 (m, 2H), 3.12 (m, 2H), 2.16−1.90 (m, 11H), 1.54−0.84 (m, 17H); ^{13}C NMR (CD_3OD) δ 177.3 (major) + 177.0 (minor), 175.6, 174.5 (major) + 174.0 (minor), 173.6 (major) + 173.4 (minor), 171.5, 169.9 (major) + 169.5 (minor), 139.8, 135.1, 130.9 (minor) + 130.8 (major), 129.5 (minor) + 129.5 (major), 128.6 (minor) + 128.6 (major), 128.3 (minor) + 128.2 (major), 118.8, 61.6 (major) + 60.9 (minor), 59.8, 58.1, 53.6, 45.8, 44.0 (minor) + 43.9 (major), 43.1 (major) + 42.9 (minor), 37.9 (major) + 37.7 (minor), 30.7, 30.6, 27.9, 27.4, 26.2, 25.8, 25.7 (major) + 25.6 (minor), 25.1 (minor) + 25.0 (major), 24.2, 22.7, 15.9, 11.3 (major) + 11.1 (minor); calcd MW 680.8; ESM 681.4. Anal. ($C_{35}H_{52}N_8O_6$) C, H, N.

Dav-His-Aib-Gly-Pro-Acp-NHBn, 46. Purification by RP-HPLC as reported above gave **46**: t_R = 13.82 min; ^{1}H NMR (CD_3OD) δ 8.73 (s, 1H), 8.33 (s, 1H), 8.22 (s, 1H), 8.14 (t, 1H, J = 6.1), 7.83 (t, 1H, J = 5.4), 7.30 (s, 1H), 7.30−7.12 (m, 7H), 4.60−4.46 (m, 2H), 4.37−4.30 (m, 2H), 3.95 (m, 2H), 3.67−3.63 (m, 2H), 3.31 (m, 2H), 2.22−1.94 (m, 11H), 1.76 (d, 4H, J = 3.1), 1.44 (s, 3H), 1.38 (s, 3H), 0.89 (m, 6H); ^{13}C NMR (CD_3OD) δ 177.4, 176.6, 175.6, 174.6, 171.4, 169.8, 140.4, 135.1, 130.7, 129.4 (minor) + 129.3 (major), 128.0, 127.8, 118.8, 68.5, 62.1, 58.1 (minor) + 58.0 (major), 53.6, 47.4 (major) + 47.1 (minor), 45.8, 44.0, 43.2, 38.8, 37.6 (minor) + 37.4 (major), 30.3, 28.0, 27.3, 26.0, 25.6, 25.6, 25.5, 25.2, 22.7; calcd MW 678.8; ESMS 678.7. Anal. ($C_{35}H_{50}N_8O_6$) C, H, N.

Dav-His-Ala-Apy-Pro-Ile-NHBn, 52. Compound **51** (94 mg, 0.1 mmol) dissolved in DCM (5 mL) was treated with a mixture of PDC (58 mg, 0.15 mmol), glacial acetic acid (10 μL) and freshly activated 3 Å molecular sieves, 1 h at room temperature. The brown suspension was filtrated over Celite and was eluted with DCM. The solvent was evaporated in vacuo. The crude product was treated with a mixture of TFA (9.5 mL), DCM (9.5 mL) and water (1 mL) for 1 h at room temperature. After evaporation of the solvent under reduced pressure, the residue was washed twice with toluene. Purification by RP-HPLC (C_{18} Bondasorb semipreparative column) using a linear gradient 100% TFA (0.1%) in water to 100% acetonitrile in 30 min (flow rate: 3 mL/min) yielded compound **53**: t_R = 14.94 min; ^{1}H NMR (CD_3OD) δ 8.78 + 8.72 (2s, 1H), 7.23 (m, 6H), 4.83 (m, 1H), 4.70 (m, 1H), 4.6−4.1 (m) + 4.38 (s, major) + 4.36 (s, minor) (6H), 3.85−3.7 (m, 1H), 3.7−3.55 (m, 1H), 3.3−3.15 (m, 1H), 3.15−3.0 (m, 1H), 2.4−1.75 (m, 7H), 1.10 (m, 1H), 1.45−1.10 (3m, 5H), 0.90 (m, 12H); ^{13}C NMR (CD_3OD) δ 176.8−176.3 (complex)140.7, 136.0, 130.4, 129.4, 129.1, 119.7 + 119.6, 65.8, 60.5, 48.1, 46.7, 44.8, 42.6, 38.9, 31.2, 29.3, 28.2, 26.9, 23.5, 16.8, 12.2; calcd MW 694.8; ESMS 694.4. Anal. ($C_{35}H_{50}N_8O_6$) H, N; C: calcd, 60.49; found, 60.01.

PPIase Assays. Recombinant human hCyp-18 expression and purification: The recombinant hCyp-18 expression system in *E. coli* was kindly provided by François Cretin (CEA-DSV/DBMS/ICH, Grenoble). It was constituted of M15 cells transformed with a pQE60 vector (Qiagen) containing an *Eco*RI-*Hin*dIII insert bearing the hCyp-18 gene without the His-Tag. A 4-L culture was grown, induced and processed as previously described.[72] Harvested cells were resuspended in a 20 mM Tris-HCl buffer, pH 7.8, containing 5 mM EDTA and a mixture of protease inhibitors and were lysed by passage through a French press. The hCyp-18-containing supernatant was concentrated by ammonium sulfate precipitation (40−75%) and dialyzed against a 20 mM Tris-HCl buffer (pH 7.8) overnight. The protein solution was passed through a DEAE-sepharose column (Pharmacia) (1.6 × 20 cm) and flow-through fractions were collected. After concentration, the hCyp-18 solution was loaded onto a SP sepharose HP column (Pharmacia) (1.6 × 20 cm) equilibrated with the 20 mM Tris-HCl buffer, pH 7.8. hCyp-18 was eluted with a 0−0.5 M NaCl gradient in the same buffer. Fractions eluted around 0.18 M NaCl contained essentially pure hCyp-18.

hCyp-18 PPIase assays:[60] hCyp-18 (16 nM) in a 35 mM Hepes buffer (pH 7.8) was incubated for 2 min with solutions of peptides in DMSO (100 μM to 100 mM; maximum volume added: 40 μL) (incubation volume: 1.98 mL). hCyp-18 activity was not modified by preincubation 2 min with up to 5% DMSO. A 20 mM solution of substrate peptide Suc-Ala-Ala-Pro-Phe-DFA[69] in TFE/0.47 M LiCl[70] (20 μL, final concentration 200 μM) was added and the absorbance variation was monitored by UV spectrophotometry at 246 mm at 10 ± 0.1 °C for 360 s.

hFKBP-12 PPIase assays:[65] Recombinant hFKBP-12 (135 nM) was incubated as reported above. A 20 mM solution of substrate peptide Suc-Ala-Leu-Pro-Phe-pNA in TFE/0.47 M LiCl (10 μL, final concentration 100 μM) was added and the absorbance variation was monitored by UV spectrophotometry at 330 mm at 10 ± 0.1 °C for 360 s.

Data Analysis. Data were recorded over a period of 6 min. After 6 min, the absorbance variation of uncatalyzed isomerization was neglectable. Data below 0.05 min were excluded

since mixture delay resulted in a random variation of absorbance. The kinetics was processed as a pseudo-first-order kinetics. Standardized apparent k_{obs} were obtained by calculation of the slopes of $k_{obs} = \ln[(A_t - A_6)/(A_{0.05} - A_6)]$, where A_t, A_6, and $A_{0.05}$ are the absorbance at times t, 0.05, and 6 min. Only data between 0.05 and 0.5 min were considered for the calculation of k_{obs}. Activities were calculated by the formula: activity (%) = $100((\text{slope}_C - \text{slope}_{uncat})/(\text{slope}_0 - \text{slope}_{uncat}))$.

Fluorimetric Determination of the Dissociation Constants. hCyp-18 (320 nM) in a 35 mM Hepes buffer (pH 7.8) was incubated for 2 min with solutions of peptides in DMSO. Variation of fluorescence was monitored using a 200-μL thermostated cell ($\lambda_{excitation}$ = 290 nm; $\lambda_{emission}$ = 324 nm). A fluorescence enhancement was observed with CsA and peptide **28,** whereas a quenching of fluorescence was recorded with Suc-AAPF-pNA.

Acknowledgment. We gratefully acknowledge SIDACTION (Grant 99014), the Agence Nationale de Recherches sur le Sida (ANRS) (Grant 70000016-01), and the Atomic Energy Commission (CEA) for financial support of this work. We are indebted to Mr. L. Demange for technical assistance for PPIase assays. We also thank Dr. M. Gondry, Dr. G. Mourier, and Dr. A. Lecoq (DIEP, CEA/Saclay) for helpful advice and discussions.

Supporting Information Available: Experimental procedures and full characterization of compounds **24**−**27**, **30**, **31**, **33**−**43**, and **47**−**51**. This material is available free of charge via the Internet at http://pubs.acs.org.

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JM9903139