# Molecular Tongs Containing Amino Acid Mimetic Fragments: New Inhibitors of Wild-Type and Mutated HIV-1 Protease Dimerization

Ludovic Bannwarth,[#,†] Albane Kessler,[⊥,†] Stéphanie Pèthe,[⊥] Bruno Collinet,[#] Naïma Merabet,[⊥] Nicole Boggetto,[#] Sames Sicsic,[⊥] Michèle Reboud-Ravaux,[#] and Sandrine Ongeri*[,⊥]

*Université de Paris−Sud XI, IFR 141, Biocis, UMR-CNRS 8076, Faculté de Pharmacie, 5 Rue J. B. Clément, F-92296 Châtenay-Malabry Cedex, France, and Laboratoire d'Enzymologie Moléculaire et Fonctionnelle, FRE 2852, Institut Jacques Monod, CNRS−Université Paris VI, 2 Place Jussieu, F-75251 Paris Cedex 05, France*

*Received May 15, 2006*

We have designed, synthesized, and evaluated the inhibitory activity and metabolic stability of new peptidomimetic molecular tongs based on a naphthalene scaffold for inhibiting HIV-1 protease dimerization. Peptidomimetic motifs were inserted into one peptidic strand to make it resistant to proteolysis. The peptidic character of the molecular tongs can be decreased without changing the way they inhibit dimerization. Mutated HIV-1 proteases are also vulnerable to dimerization inhibitors, and the multimutated protease ANAM-11 is twice as sensitive to the inhibitor compared to wild-type protease. Thus, the metabolic stability of antidimeric molecular tongs can be increased without compromising their ability to inhibit wild-type and mutated HIV-1 proteases in vitro.

## Introduction

HIV-1 protease (PR) is an important target for anti-AIDS drugs because its inhibition results in the production of uninfectious virus.[1,2] The PR inhibitors used in highly active antiretroviral therapy (HAART) act in conjunction with two inhibitors of reverse transcriptase to reduce the virus load and increase the number of CD4 lymphocytes in AIDS patients. The most recent inhibitors, like tripanavir, have much less peptidic character than the first commercially available inhibitor saquinavir.[3−6] However, the development of cross-resistance to protease inhibitors is a serious limitation in long-term treatment of AIDS patients.[7,8] Several mutations located within or outside the active site of PR have been observed and shown to lower the affinity of the enzyme for inhibitors while not interfering with its enzymatic profile. Although new peptidomimetic and nonpeptidomimetic PR inhibitors are being developed by the pharmaceutical industry to overcome this resistance, alternative strategies for controlling HIV replication by inhibiting PR are required. One possibility is to design inhibitors that prevent the dimerization of PR or favor the dissociation of the dimer because the enzyme is a homodimer. Protein−protein interactions in the dimer include the antiparallel $\beta$-sheet formed by interdigitation of the N- (residues 1−4) and C- (residues 96−99) terminal strands of monomers (Figure 1), which contributes close to 75% of the total Gibbs free energy of dimerization.[9,10] This antiparallel $\beta$-sheet is an attractive target for overcoming resistance to clinical drugs because its amino acid residues are highly conserved in most HIV-1 and HIV-2 isolates.[11−13] Blocking the formation of the homodimer or disrupting it is an effective way of inhibiting protease activity.[14] Thus, all known inhibitors of PR dimerization developed by rational design target this region. C- and N-terminal mimetic peptides were the first dimerization inhibitors to be developed.[15−17] Suitable variation of the nature of the peptidic sequence and addition of a lipophilic group to the N-end favor targeting of the antiparallel $\beta$-sheet and improve the efficiency of inhibitors.[18,19] The interface peptides can also be cross-linked with flexible,[20] semirigid,[21] or rigid spacers.[22,23] A bicyclic guanidinium has also been successfully introduced between a peptidic chain and a hydrophobic group.[24] Our contribution to the antidimer strategy was the synthesis of molecular tongs based on a conformationally constrained scaffold attached to two peptidic strands through two carboxypropyl links. Inhibitors that use constrained scaffolds of naphthalene (compound **1**, Figure 1) or quinoline led to efficient antidimers ($K_{id}$ = 80 nM).[22,23] The asymmetrical inhibitors (two different peptidic arms) are more efficient than symmetrical ones (two identical arms).[23] This report describes the synthesis, enzyme inhibitory activity against wild-type and mutated HIV-1 proteases, and metabolic stability of the new asymmetrical molecular tongs **2**−**6** containing amino acid mimetic fragments in one strand with the other peptidic strand being VLV-OMe (Figure 2). VLV-OMe is used because our previous studies showed that this sequence led to the best dimerization inhibitor.[22,23] These new molecular tongs **2**−**6** were designed to have less peptidic character and consequently to be less susceptible to proteases in vivo. The peptidic character of a molecule can be decreased by replacing one or more amino acids with groups that mimic their hydrogen-bonding properties. The amino acids of the molecular tongs that inhibited PR are believed to be involved in hydrogen bond interactions with the N- and C-termini of one PR monomer, favoring the formation of a $\beta$-sheet structure and mimicking the four-strand $\beta$-sheet structure of the PR dimer (Figure 1).[22,23] Consequently, the groups replacing amino acids should ideally provide the same array of hydrogen-bonding groups as one edge of a peptidic $\beta$-strand. In literature, Nowick and Sanderson have introduced a 5-amino-2-methoxybenzamide group[25] and a 3-amino-6-methylpyridin-2(1*H*)-one group[26] to potentially form hydrogen bonds such as those of amino acids. We therefore designed new molecular tongs with two amino acids of one strand replaced by a peptidomimetic motif. The peptidomimetic Nowick motif was inserted into molecular tongs **2** and **6**, and the peptidomimetic Sanderson motif was placed in molecular tongs **3** and **4** (Figure 2). In molecule **5**, the peptidomimetic Sanderson motif was simplified by omitting the methyl group in position 6 of the

* To whom correspondence should be addressed. Phone: 33(0)-146835743. Fax: 33(0)146835740. E-mail: sandrine.ongeri@cep.u-psud.fr.
[#] CNRS−Université de Paris VI.
[†] These authors contributed equally.
[⊥] Université de Paris−Sud XI.

10.1021/jm060576k CCC: $33.50 
Published on Web 07/06/2006

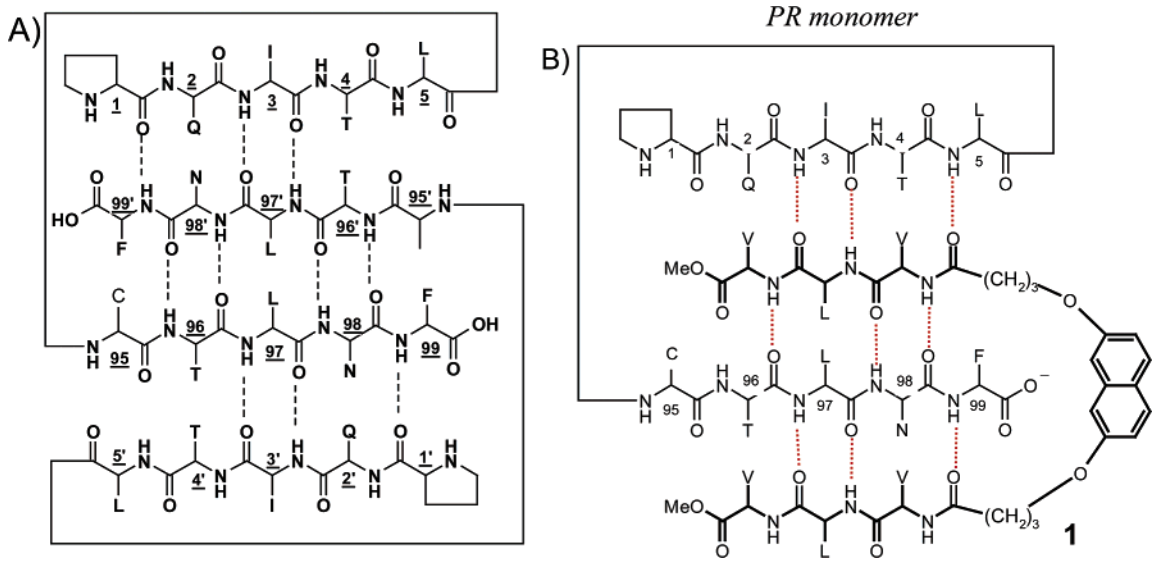



**Figure 1.** (A) Diagram of the HIV-1 protease $\beta$-sheet. (B) Putative complex formed between HIV-1 monomer and compound **1**.

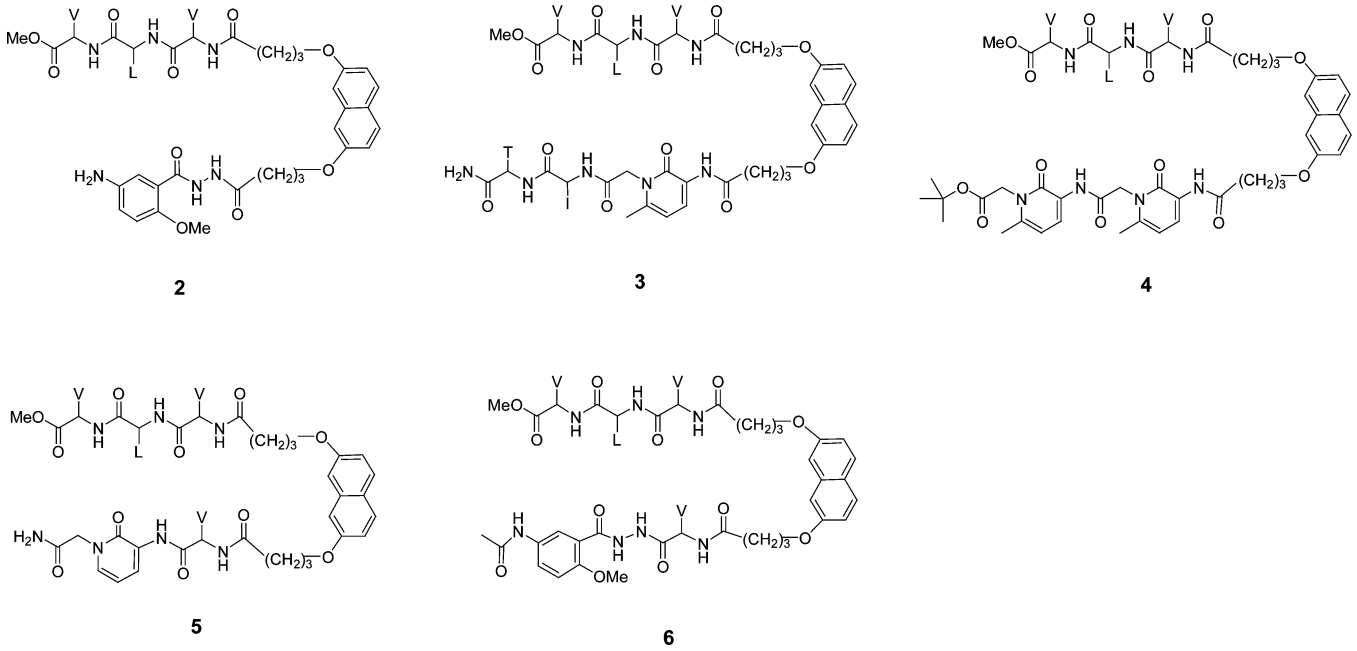



**Figure 2.** Molecular tongs **2**−**6**.

3-aminopyridin-2(1*H*)-one (Figure 2). We also identified the most suitable position of the peptidomimetic unit within the strand for active molecular tongs. Two cases were examined: first, the mimic unit was attached directly to the carboxypropyl link (molecules **2**, **3**, and **4)**; second, it was linked through a Val unit (compounds **5** and **6)**. The results of building several possible complexes using the Sybyl 7.0 molecular modeling tools indicated that forming the postulated $\beta$-sheet structure between the N- and C-termini of one PR monomer and molecule **5** or molecule **6** (Figure 3) is energetically possible, since the correct array of alternating 10-member and 14-member rings of hydrogen bonds is still present after minimization. Conversely, no stable $\beta$-sheet structure was formed with molecules **2**−**4**. We synthesized and tested molecules **2**−**6** to confirm these molecular models.

## Chemistry

The peptidomimetic strand **11** was obtained in four steps from the Boc-protected 5-amino-2-methoxybenzohydrazide **7** described by Nowick (Scheme 1).[25e] The protected benzoic acid hydrazide **7** was acylated and the hydrazide **8** deprotected. The resulting compound **9** was coupled to Z-valine using HBTU and HOBT as coupling agents, with a good yield to give the Z-protected compound **10**, which was deprotected by hydrogenolysis to give **11** (Scheme 1). The peptidomimetic strand **14** was obtained by condensation of N-protected (3-amino-6-methyl-2-oxopyridin-1(2*H*)-yl)acetic acid **12**[26] with the dipeptide Ile-Thr-$NH_2$ using HBTU and by deprotection of the resulting compound **13** (Scheme 2). Strand **17** containing two peptidomimetic units was obtained by condensation of the N-protected Sanderson motif **12**[26] with the *t*-Bu ester **15**[26] of this same motif in the presence of HBTU and by the N-deprotection of the resulting compound **16** (Scheme 2). The peptidomimetic **22** was synthesized from commercially available 3-nitropyridin-2(1*H*)-one **18**, which was successively N-alkylated by bromoacetamide, hydrogenated into compound **20**, condensed with Z-Val in the presence of EDCI and HOBT, and N-deprotected by hydrogenolysis (Scheme 3). The molecular tong **2** was synthesized

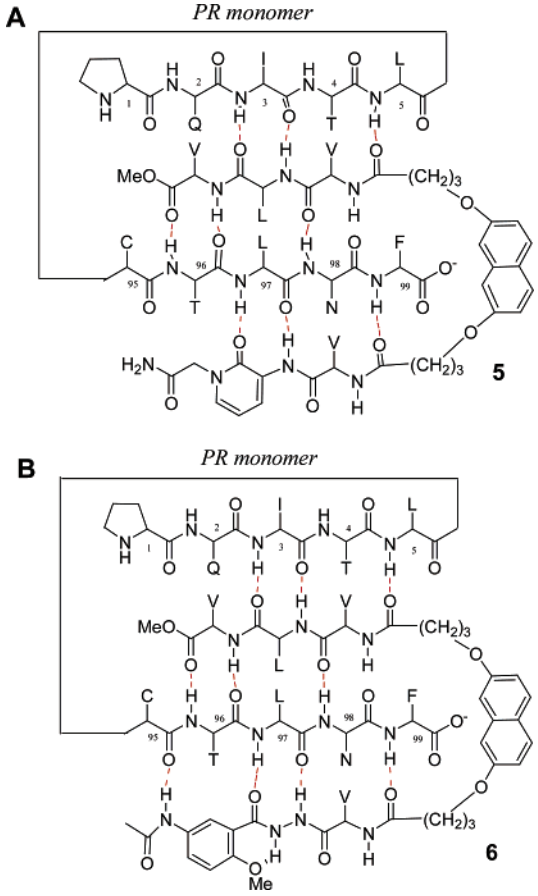
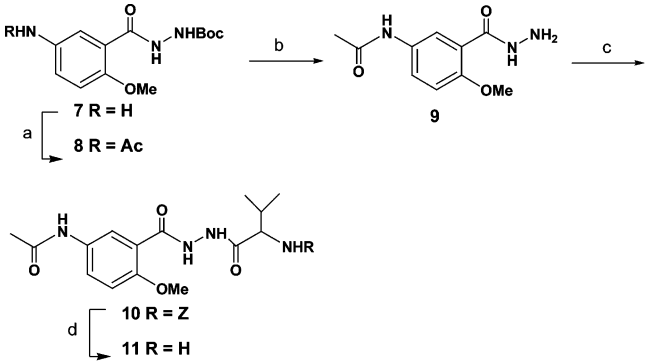



**Figure 3.** Putative complexes between HIV-1 protease monomer and molecular tongs **5** and **6** based on molecular modeling.

**Scheme 1.** Synthesis of Peptidomimetic Containing Strand **11**[a]



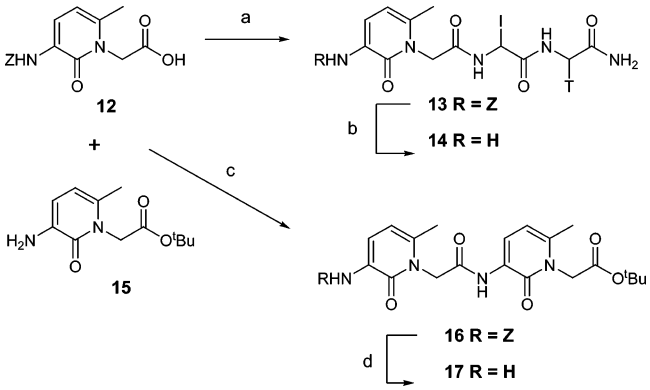

[a] (a) $Ac_2O$, THF, 40 min, 60 °C; (b) TFA, $CH_2Cl_2$, 3 h, room temp; (c) Z-V-OH, HBTU, HOBT, DIPEA, DMF, 28 h, room temp; (d) $H_2$, 10% Pd/C, HCl/MeOH, DMF, room temp, 3 h.

by condensing **23**[23] with peptidomimetic **25**, obtained by N-deprotection of Nowick derivative **24**,[25e] followed by hydrogenation of the nitro group of the intermediate **26** (Scheme 4). Finally, the molecular tongs **3**−**6** were synthesized directly, in the presence of HBTU, by condensation of **23**[23] with peptidomimetic motifs (unprotected **17**) or with strands containing peptidomimetic motifs (**11**, **14**, or **22**) (Scheme 4).

## Biological Results and Discussion

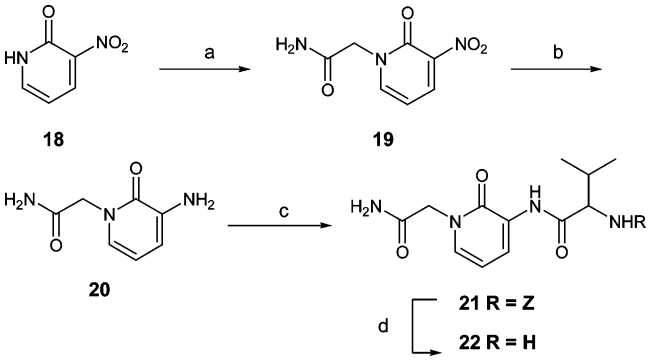
The inhibitory activities of compounds **2**−**6** were assayed against recombinant wild-type PR at pH 4.7 and 30 °C using a fluorometric assay.[22,23] Compound **5** was also assayed against six recombinant mutated proteases. The poor solubility of compound **2** in the experimental conditions for activity assays

**Scheme 2.** Synthesis of Peptidomimetic Containing Strands **14** and **17**[a]



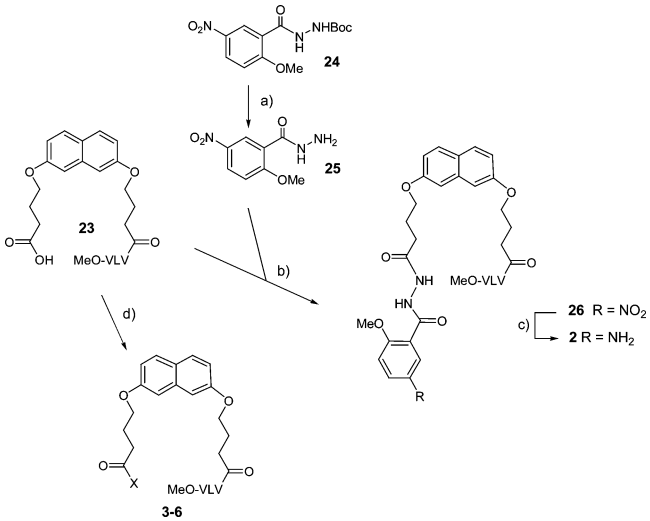

[a] (a) H-IT-$NH_2$, HBTU, $Et_3N$, DMF, 48 h, room temp; (b) $H_2$ 10% Pd/C, MeOH, 20 h, room temp; (c) HBTU, $Et_3N$, DMF, 24 h, room temp; (d) $H_2$, 10% Pd/C, MeOH, 24 h, room temp.

**Scheme 3.** Synthesis of Peptidomimetic Containing Strand **22**[a]




[a] (a) $BrCH_2CONH_2$, $K_2CO_3$, THF, 16 h, 60 °C; (b) $H_2$, 10% Pd/C, MeOH, 4 h, room temp; (c) Z-V-OH, EDCI, HOBT, NMM, DMF, 48 h, room temp; (d) $H_2$, 10% Pd/C, MeOH, 20 h, room temp.

**Scheme 4.** Synthesis of Molecular Tongs **2**−**6**




[a] (a) 3.5 N HCl/MeOH, 24 h, room temp; (b) HBTU, HOBt, $Et_3N$, DMF, 24 h, room temp; (c) $H_2$, 10% Pd/C, MeOH, 24 h, room temp; (d) HBTU, $Et_3N$ or DIPEA, X = **11**, **14**, **17**, or **22**, DMF, 24−48 h, room temp.

(100 mM sodium acetate, 1 mM EDTA, 1 M NaCl, 3% v/v DMSO) prevented its study at concentrations higher than 5 $\mu$M (28% inhibition). The mechanism of inhibition of PR by other compounds was assessed using Zhang−Poorman kinetic analysis in which plots of $[E]_0/\sqrt{v_i}$ vs $\sqrt{v_i}$, with $\sqrt{v_i} = k_{exp}[S]_0$, are constructed[16] in order to discriminate between inhibition of

**Table 1.** In Vitro Inhibition of Wild-Type and Mutated HIV-1 Proteases by Molecular Tongs (30 °C and pH 4.7) and Comparison with Inhibition by Therapeutical Antiproteases[a]

| compd | wild-type (WT) or mutated protease[b] | $K_{ic}$ ($\mu$M)[c] | $K_{id}$ ($\mu$M)[d] | $K_{id,mut}/K_{id,WT}$ | clinical antiprotease | $K_{i,mut}/K_{i,WT}$ |
|---|---|---|---|---|---|---|
| **1** | WT | | 0.56[22] | | | |
| **2** | WT | I[e] | I[e] | | | |
| **3** | WT | 9.7 | | | | |
| **4** | WT | 8.4 | | | | |
| **5** | WT | | 0.4 | | | |
| **5** | **D30N** | | 0.5 | 1.2 | nelfinavir[27] | 6 |
| **5** | **I50V** | | 0.1 | 0.25 | amprenavir[29] | 83 |
| **5** | **V82A** | | 0.24 | 0.6 | ritonavir[28] | 15 |
| **5** | **G48V**/L90M | | 0.5 | 1.25 | saquinavir[30] | 1000 |
| **5** | **MDR-HM** | | 4.8 | 12 | nelfinavir[31] | 210 |
| **5** | **MDR-HM** | | 4.8 | 12 | amprenavir[31] | 85 |
| **5** | **MDR-HM** | | 4.8 | 12 | indinavir[31] | 200 |
| **5** | **MDR-HM** | | 4.8 | 12 | saquinavir[31] | 2000 |
| **5** | **MDR-HM** | | 4.8 | 12 | ritonavir[31] | 1500 |
| **5** | **ANAM-11** | | 0.2 | 0.5 | nelfinavir[32] | 2840 |
| **5** | **ANAM-11** | | 0.2 | 0.5 | indinavir[32] | 2030 |
| **5** | **ANAM-11** | | 0.2 | 0.5 | saquinavir[32] | 4200 |
| **5** | **ANAM-11** | | 0.2 | 0.5 | ritonavir[32] | 78000 |
| **6** | WT | | 0.2 | | | |

[a] Standard errors of initial rates are less than 5%. [b] Mutations in the active site are in bold. [c] Competitive active site inhibition. [d] Dimerization inhibition. [e] 28% inhibition at 5 $\mu$M.

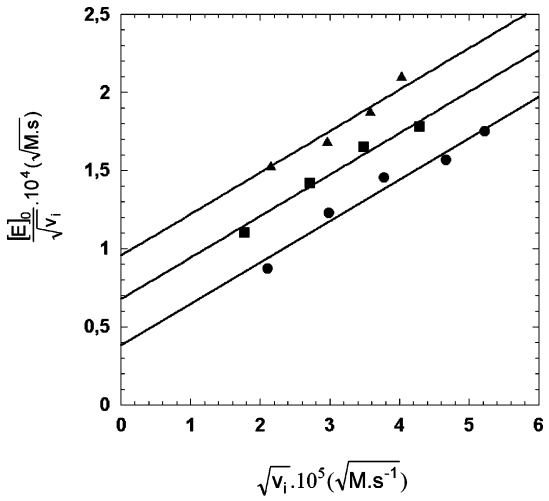



**Figure 4.** Plots of $[E]_0/\sqrt{v_i}$ vs $\sqrt{v_i}$ for the hydrolysis of the fluorogenic substrate DABCYL-$\gamma$-abu-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Gln-EDANS by HIV-1 PR WT at pH 4.7 and 30 °C in the absence (●) and presence of 1 $\mu$M (■) and 2 $\mu$M (▲) compound **5**.

dimerization alone (parallel lines), competitive inhibition (altered slopes and unaltered *y*-axis intercept), and mixed inhibition (altered slopes and altered *y*-axis intercepts). In pure dimerization inhibition, the inhibitor binds to the interface region, whereas a competitive inhibitor binds to the active site and a mixed inhibitor may bind to the active site as well as to the interfacial region. The results are summarized in Table 1. Convergent lines were obtained for compounds **3** and **4**, demonstrating that they act as active-site competitive inhibitors; parallel lines were obtained with compounds **5** and **6,** which is consistent with pure dimerization inhibition (Figure 4). This result indicates that replacing the tripeptide VLV (compound **1**) with a pseudopeptidic strand composed of valine linked to the demethylated Sanderson motif (compound **5**) or the Nowick motif (compound **6**) results in an antidimer inhibitor that is still fully active against wild-type PR ($K_{id}$ = 0.4 $\mu$M for **5** and $K_{id}$ = 0.2 $\mu$M for **6**, compared to $K_{id}$ = 0.56 $\mu$M for **1**). Thus, the Nowick and demethylated Sanderson peptidomimetic units can be used to replace amino acids in the structures of the previously described molecular tongs. The results are in agreement with molecular modeling, indicating that the antidimer property depends on the position of the mimic unit in the peptidomimetic strand. They suggest that the flexibility of valine is required for the formation of the hydrogen bonds in a four-strand antiparallel $\beta$-sheet, implicating the N- and C-termini of the PR monomer. It was essential to evaluate the action of our dimerization inhibitors against the drug-resistant PR mutants we have produced. Some active-site mutations are known to preferentially affect the therapeutical antiproteases that bind to the active site: D30N (nelfinavir),[27] V82A (ritonavir),[28] I50V (amprenavir).[29] The double-point mutation G48V/L90M affects saquinavir.[30] Mutation L90M is located at the terminal region, close to the dimerization interface. Two multidrug-resistant mutants were also used. The mutant MDR-HM[31] contains six (L10I/M46I/I54V/V82A/I84V/L90M) amino acid mutations located within and outside the active site of the enzyme, while ANAM-11[32] has 11 mutations (L10I/M36I/S37D/M46I/R57K/L63P/A71V/G73S/I84V/L90M/I93L). Remarkably, the new pseudopeptidic molecular tong **5** behaved as an antidimer inhibitor against single and double mutated proteases and against the two multidrug-resistant proteases (Table 1). It inhibited mutants D30N and G48V/L90M as actively as wild-type PR ($K_{id,mut}/K_{id,WT} \approx 1.2$) and was even more active against some mutated proteases than against wild-type PR ($K_{id,mut}/K_{id,WT}$ = 0.6 and 0.25 for V82A and I50V, respectively). At the opposite, important decreases in in vitro potency against the same mutated proteases were observed for active site inhibitors (Table 1) whose activities were reduced 15-fold (ritonavir/V82A),[28] 6-fold (nelfinavir/D30N),[27] 83-fold (amprenavir/I50V),[29] or even 1000-fold (saquinavir/ G48V/L90M).[30] Similar results were obtained with multidrug-resistant proteases. For example, the affinity of ANAM-11 protease for compound **5** was 2-fold greater ($K_{id}$ = 200 nM) than that of wild-type PR ($K_{id}$ = 400 nM). In contrast, the affinities of this mutated protease for indinavir, nelfinavir, saquinavir, and ritonavir were about 2030, 2840, 4200, and 78 000 times lower than that of wild-type PR.[32] This demonstrates that the peptidomimetic molecular tong **5** still acts as an efficient inhibitor of the dimerization of mutated proteases. It inhibits in vitro 87-fold more actively ANAM-11 protease than does ritonavir. The initial hypothesis suggesting that inhibitors of PR dimerization are poorly sensitive to mutations is confirmed, as previously proposed by Chmielewski and collaborators who developed dimerization inhibitors of HIV-1 PR by cross-linking interface peptides with flexible spacers.[33] Finally, the stability of the peptide (**1**) and peptidomimetic (**5** and **6**) molecular tongs in RPMI culture medium containing 20% fetal calf serum was studied. The half-life of compound **1** was about 28 h and that of **5** about 41 h; no significant degradation of compound **6** was observed after 33 h of incubation. The results suggest that the metabolic stability has been improved by inserting peptidomimetics into one arm of the molecular tong.

## Conclusion

Our previous finding that molecular tongs are inhibitors of the PR interface[22,23] prompted us to create proteolysis-resistant inhibitors by replacing the peptidics strands with peptidomimetics. We replaced two amino acids in one strand of the naphthalene-based molecular tongs by a peptidomimetic to produce good inhibitors of PR dimerization. The resistance to hydrolysis of these new molecular tongs is increased without modifying their inhibitory potency. One of these dimerization inhibitors was also evaluated against proteases with 1, 2, 6, and

11 point mutations; it was as active (and in some cases more active) on mutated proteases as on wild-type PR. This suggests that peptidomimetic molecular tongs are candidates to successfully overcome the resistances presently encountered with classical protease inhibitors. Our results also provide new information on the mechanism of action of molecular tongs. We postulated that the molecular tongs inhibit the dimerization of PR via the formation of a four-strand antiparallel $\beta$-sheet with one monomer of the protease.[22,23] This study provides new arguments for this because it confirms the molecular modeling study. In contrast to molecules **2**−**4**, hydrogen bonds can be formed in molecular tongs **5** and **6** between the carbonyl group of each carboxypropyl group and the N-terminal NH of the Leu-5 and the NH of the C-terminal Phe-99, respectively (Figures 1 and 3).

Further studies on inhibitors of HIV-1 protease dimerization containing peptidomimetics will focus on the synthesis of molecular tongs with quinoline as the constrained scaffold, since quinoline-based tongs with peptidic strands are also interfacial inhibitors of the HIV-1 PR interface,[23] and on the measurement of their antiviral efficacy in cell culture assays.

## Experimental Section

**Synthesis.** The usual solvents were purchased from commercial sources. Dimethylformamide (DMF) was distilled on $CaSO_4$. Tetrahydrofuran (THF) was distilled on sodium/benzophenone, and acetonitrile was distilled on $CaCl_2$. TLC was performed on silica gel 60F-250 (0.26 mm thickness) plates. The plates were visualized with UV light (254 nm) or with a 3.5% solution of phosphomolybdic acid in ethanol or with a solution of ninhydrin in ethanol. Liquid chromatography was performed on Merck 60 silica gel (230−400 mesh). 2-Hydroxy-3-nitropyridine **18**, 2-bromoacetamide, protected amino acids, *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU), 1-hydroxybenzotriazol (HOBT), and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDCI) were purchased from commercial sources. H-Val-Leu-Val-OMe, H-Ile-Thr-$NH_2$, 4-[7-(3-carboxypropoxy)naphthalen-2-yloxy]butyryl-Val-Leu-Val-OMe **23**,[23] the Boc protected 5-amino-2-methoxybenzohydrazide **7**,[25e] the Boc protected 2-methoxy-5-nitrobenzohydrazide **24**,[25e] 3-benzyloxycarbonylamino-6-methyl-1-methylenecarboxy-2-pyridinone **12**,[26] and 3-amino-6-methyl-1-*tert*-butylmethylenecarboxy-2-pyridinone **15**[26] were prepared according to published methods. Melting points were determined on a Kofler melting point apparatus. NMR spectra were performed on a Bruker AMX 200 ($^1$H, 200 MHz; $^{13}$C, 50 MHz) or a Bruker AVANCE 400 ($^1$H, 400 MHz; $^{13}$C, 100 MHz). Unless otherwise stated, $CDCl_3$ was used as the solvent. Chemical shifts $\delta$ are in ppm, and the following abbreviations are used: singlet (s), doublet (d), doublet doublet (dd), triplet (t), quadruplet (q), multiplet (m), broad doublet (bd), broad triplet (bt), and broad singlet (bs). Mass spectra were obtained using a Bruker Esquire electrospray ionization apparatus. Element analyses (C, H, N) were performed on a Perkin-Elmer CHN analyzer, model 2400, at the Microanalyses Service of the Faculty of Pharmacy at Châtenay-Malabry (France).

***N*′-(5-Acetylamino-2-methoxybenzoyl)hydrazinecarboxylic Acid *tert*-Butyl Ester 8.** Compound **7** (1.1 g, 3.9 mmol) was dissolved in dry THF (15 mL), and the solution was heated to 60 °C. Acetic anhydride (1.3 mL, 13.7 mmol) was added dropwise to this solution. The reaction mixture was stirred for 40 min at 60 °C. THF was evaporated under reduced pressure, and the crude product was dried under vacuum to yield a white powder: mp 211−213 °C (1.24 g, 99% yield). This crude product was used without any further purification in the course of the synthesis. $^1$H NMR (DMSO-$d_6$) $\delta$ 9.9 (s, 1H), 9.6 (s, 1H), 8.9 (bs, 1H), 7.9 (s, 1H), 7.7 (d, $J$ = 8.4 Hz, 1H), 7.05 (d, $J$ = 8.4 Hz, 1H), 3.85 (s, 3H), 2.0 (s, 3H), 1.40 (s, 9H); $^{13}$C NMR (DMSO-$d_6$) $\delta$ 168.3, 165.3, 155.6, 153.1, 132.9, 123.6, 121.7, 112.6, 79.4, 53.3, 28.4, 24.1

***N*-(3-Hydrazinocarbonyl-4-methoxyphenyl)acetamide 9.** Compound **8** (200 mg, 0.62 mmol) was suspended in $CH_2Cl_2$ (20 mL). TFA (1.2 mL, 15.5 mmol) was then added dropwise at room temperature. The reaction mixture was stirred under argon atmosphere at room temperature for 3 h. Solvent was evaporated under reduced pressure to yield a pale-yellow solid (210 mg, quantitative yield). This crude product was used without any further purification in the course of the synthesis. $^1$H NMR (MeOD-$d_4$) $\delta$ 8.2 (d, $J$ = 2.4 Hz, 1H), 7.7 (dd, $J$ = 9.0 Hz, 2.4 Hz, 1H), 7.2 (d, $J$ = 9.0 Hz, 1H), 4.0 (s, 3H), 2.1 (s, 3H); $^{13}$C NMR (MeOD-$d_4$) $\delta$ 175.4, 170.1, 166.4, 154.1, 131.8, 125.1, 122.8, 111.7, 53.2, 22.0.

**{1-[*N*′-(5-Acetylamino-2-methoxybenzoyl)hydrazinocarbonyl]-2-methylpropyl}carbamic Acid Benzyl Ester 10.** Compound **9** (209 mg, 0.62 mmol) and Z−V−OH (156 mg, 0.62 mmol) were dissolved in DMF (20 mL). DIPEA (0.6 mL, 3.1 mmol) was then added. HBTU (259 mg, 0.68 mmol) and HOBt (93 mg, 0.68 mmol) were added to the reaction flask. The reaction mixture was stirred under argon atmosphere atmosphere at room temperature for 24 h. DMF was evaporated under reduced pressure and the residue was dissolved in EtOAc (150 mL), washed with aqueous citric acid 5% (50 mL), aqueous $Na_2CO_3$ 10% (50 mL), and brine (50 mL). The organic phase was dried over $MgSO_4$ and concentrated under reduced pressure. The crude solid was triturated in EtOAc, filtered and dried under vacuum to yield a pale yellow powder: mp 227−229°C (201 mg, 71% yield); $^1$H NMR (DMSO-$d_6$) $\delta$ 10.3 (bs, 1H), 9.9 (bs, 2H), 7.9 (d, $J$ = 2.6 Hz, 1H), 7.8 (dd, $J$ = 9 Hz, 2.6 Hz, 1H), 7.3 (m, 5H), 7.1 (d, $J$ = 9.0 Hz, 1H), 5.0 (s, 2H), 4.0 (m, 1H), 3.8 (s, 3H), 2.0 (s, 3H), 1.9 (m, 1H), 0.9 (m, 6H); $^{13}$C NMR (DMSO-$d_6$) $\delta$ 169.3, 167.8, 155.9, 152.5, 149.4, 132.7, 132.5, 128.2, 127.6, 127.5, 124.8, 123.2, 121.2, 112.3, 65.3, 58.6, 56.1, 30.4, 23.6, 19.0, 18.1. Anal. ($C_{23}H_{28}N_4O_6\cdot 0.4H_2O$) C, H, N.

***N*-{3-[*N*′-(2-Amino-3-methylbutyryl)hydrazinocarbonyl]-4-methoxyphenyl}acetamide 11.** To a solution of **10** (140 mg, 0.62 mmol) in DMF (10 mL) was added Pd/C (28 mg, 20% mass). The reaction flask was purged three times with hydrogen, and stirring was maintained under hydrogen atmosphere at room temperature for 3 h. The mixture was filtered through a mixture of Celite and silica, and the cake was washed thoroughly with DMF (200 mL). The filtrate was evaporated to dryness, and the residue was rinsed with MeOH (20 mL) to yield the crude product as a white powder: mp 193−195 °C (67 mg, 67% yield); $^1$H NMR (DMSO-$d_6$) $\delta$ 10.1 (s, 1H), 10.0 (s, 1H), 9.8 (s, 1H), 8.3 (bs, 2H), 7.9 (d, $J$ = 2.7 Hz, 1H), 7.7 (dd, $J$ = 9.0 Hz, 2.7 Hz, 1H), 7.1 (d, $J$ = 9.0 Hz, 1H), 3.8 (s, 3H), 3.6 (d, $J$ = 5.7 Hz, 1H), 2.1 (m, 1H), 2.0 (s, 3H), 1.0 (d, $J$ = 6.8 Hz, 6H); $^{13}$C NMR (DMSO-$d_6$) $\delta$ 168.4, 166.4, 163.8, 152.9, 133.0, 121.5, 112.8, 56.6, 34.4, 30.2, 24.1, 18.5, 18.2

**(1-{[1-(1-Carbamoyl-2-hydroxypropylcarbamoyl)-2-methylbutylcarbamoyl]methyl}-6-methyl-2-oxo-1,2-dihydropyridin-3-yl)carbamic Acid Benzyl Ester 13.** To a solution of **12** (150 mg, 0.47 mmol) in DMF (3 mL) was added HBTU (230 mg, 0.52 mmol) and $Et_3N$ (0.05 mL, 0.52 mmol). The mixture was stirred for 20 min at room temperature. I-T-$NH_2$ (110 mg, 0.47 mmol) was then added, and the mixture was stirred at room temperature for 48 h. DMF was evaporated under reduced pressure, and the residue was dissolved in $CH_2Cl_2$ (50 mL), washed with $H_2O$ (10 mL), aqueous 1 N HCl (10 mL), and 20% aqueous NaOH (10 mL). The organic phase was dried over $MgSO_4$, filtrated, and concentrated under reduced pressure. Trituration of the residue in $Et_2O$ (20 mL) and filtration afforded the pure product as an off-white powder: mp 249−251 °C (150 mg, 60% yield); $^1$H NMR (DMSO-$d_6$) $\delta$ 8.4 (d, $J$ = 8.5 Hz, 1H), 8.3 (s, 1H), 7.7 (m, 2H), 7.3 (m, 5H), 7.0 (s, 2H), 6.1 (d, $J$ = 9.4 Hz, 1H), 5.1 (s, 2H), 4.9 (m, 2H), 4.7 (d, $J$ = 8.9 Hz, 1H), 4.2 (t, $J$ = 7.9 Hz, 1H), 4.1 (m, 1H), 4.0 (m, 1H), 2.2 (s, 3H), 1.8 (m, 1H), 1.4 (m, 1H), 1.1 (m, 1H), 1.0 (d, $J$ = 3.4 Hz, 3H), 0.8 (m, 6H); $^{13}$C NMR (DMSO-$d_6$) $\delta$ 171.0, 170.0, 167.0, 166.0, 157.5, 140.0, 136.5, 129.0, 125.0, 123.0, 106.5, 67.0, 66.0, 59.0, 58.0, 48.0, 37.0, 25.0, 21.0, 16.0, 12.0. Anal. ($C_{26}H_{35}N_5O_7$) C, H, N

**2-[2-(3-Amino-6-methyl-2-oxopyridin-1(2*H*)-yl)acetylamino]-3-methylpentanoic Acid (1-Carbamoyl-2-hydroxypropyl)amide 14.** To a solution of **13** (100 mg, 0.19 mmol) in MeOH (5 mL) was added Pd/C (50 mg, 50% mass). The reaction flask was purged three times with hydrogen, and stirring was maintained under

hydrogen atmosphere at room temperature for 24 h. The mixture was filtered through a mixture of Celite and silica, and the cake was washed thoroughly with MeOH (100 mL). The filtrate was evaporated to dryness to yield a colorless oil. Trituration in $Et_2O$ (20 mL) yielded an off-white powder: mp 104−106 °C (53 mg, 70% yield); $^1$H NMR (DMSO-$d_6$) $\delta$ 8.4−8.2 (m, 3H), 7.7 (m, 2H), 7.0 (s, 2H), 6.0 (d, $J$ = 9.4 Hz, 1H), 4.8−4.7 (m, 3H), 4.2 (t, $J$ = 8.4 Hz, 1H), 4.1 (m, 1H), 4.0 (m, 1H), 2.2 (s, 3H), 1.8 (m, 1H), 1.4 (m, 1H), 1.1 (m, 1H), 1.0 (d, $J$ = 3.4 Hz, 3H), 0.8 (m, 6H); $^{13}$C NMR (DMSO-$d_6$) $\delta$ 171.5, 170.0, 167.0, 158.0, 140.0, 126.0, 123.0, 106.0, 67.0, 58.0, 57.0, 47.0, 38.0, 25.0, 20.0, 15.0, 11.0. Anal. ($C_{18}H_{29}N_5O_5$) C, H, N

**{3-[2-(3-Benzyloxycarbonylamino-6-methyl-2-oxopyridin-1(2*H*)-yl)acetylamino]-6-methyl-2-oxopyridin-1(2*H*)-yl}acetic Acid *tert*-Butyl Ester 16.** To a solution of **12** (100 mg, 0.36 mmol) in DMF (3 mL) was added HBTU (153 mg, 0.40 mmol) and $Et_3N$ (0.14 mL, 0.40 mmol). The mixture was stirred for 20 min at room temperature. Amine **15** (86 mg, 0.36 mmol) was then added, and the mixture was stirred at room temperature for an additional 24 h. DMF was evaporated under reduced pressure, and the residue was dissolved in $CH_2Cl_2$ (50 mL), washed with $H_2O$ (20 mL), aqueous 1 N HCl (20 mL) and 20% aqueous NaOH (20 mL). The organic phase was dried over $MgSO_4$, filtered, and concentrated under reduced pressure. Trituration of the residue in $Et_2O$ (20 mL) and filtration afforded the pure product as an off-white powder: mp 237−239 °C (58 mg, 30% yield); $^1$H NMR (DMSO-$d_6$) $\delta$ 8.3 (s, 1H), 8.7 (s, 1H), 8.1 (d, $J$ = 7.8 Hz, 1H), 7.8 (d, $J$ = 7.8 Hz, 1H), 7.4−7.3 (m, 5H), 6.2 (d, $J$ = 7.9 Hz, 2H), 5.2 (s, 2H), 5.0 (s, 2H), 4.8 (s, 2H), 2.2 (s, 6H), 1.4 (s, 9H); $^{13}$C NMR (DMSO-$d_6$) $\delta$ 168.0, 167.0, 158.1, 154.0, 140.5, 137.0, 128.5−127.0, 126.0, 123.0, 124.1, 106.5, 82.0, 66.5, 48.5, 47.0, 28.3, 20.0. Anal. ($C_{28}H_{32}N_4O_7\cdot 0.15H_2O$) C, H, N

**{3-[2-(3-Amino-6-methyl-2-oxopyridin-1(2*H*)-yl)acetylamino]-6-methyl-2-oxopyridin-1(2*H*)-yl}acetic Acid *tert*-Butyl Ester 17.** To a solution of **16** (200 mg, 0.37 mmol) in MeOH (5 mL) was added Pd/C (100 mg, 50% mass). The reaction flask was flushed three times with hydrogen, and stirring was maintained under hydrogen atmosphere at room temperature for 24 h. The mixture was filtered through a mixture of Celite and silica, and the cake was washed thoroughly with MeOH (200 mL). The filtrate was evaporated to dryness to yield a pale-yellow oil. Trituration in $Et_2O$ (20 mL) yielded a pale-yellow powder: mp 144−150 °C (98 mg, 66% yield); $^1$H NMR (DMSO-$d_6$) $\delta$ 8.2 (m, 4H), 6.5 (d, $J$ = 7.2 Hz, 1H), 6.2 (d, $J$ = 7.7 Hz, 1H), 6.0 (d, $J$ = 7.3 Hz, 1H), 5.0 (s, 2H), 4.8 (s, 2H), 2.3 (s, 3H), 2.2 (s, 3H), 1.4 (s, 9H); $^{13}$C NMR (DMSO-$d_6$) $\delta$ 168.0, 167.0, 158.0, 140.0, 126.0, 123.0, 124.0, 105.0, 80.0, 47.8, 28.5, 20.5. Anal. ($C_{20}H_{26}N_4O_5\cdot 0.15H_2O$) C, H, N

**2-(3-Nitro-2-oxopyridin-1(2*H*)-yl)acetamide 19.** To a solution of 3-nitropyridin-2(1*H*)-one **18** (0.35 g, 2.5 mmol) and 2-bromoacetamide (0.48 g, 3.0 mmol) in dry THF (12 mL) was added $K_2CO_3$ (0.41 g, 3.0 mmol). The reaction mixture was refluxed with stirring for 16 h. The resulting precipitate was filtered, and the yellow solid was washed with MeOH (20 mL) and dried overnight in a desiccator. Trituration in hot ethanol (20 mL) and filtration afforded the pure product as a yellow powder: mp 200−202 °C (405 mg, 82% yield); $^1$H NMR (DMSO-$d_6$) $\delta$ 8.2 (dd, $J$ = 2.0, 7.7 Hz, 1H), 7.9 (dd, $J$ = 2.0, 6.6 Hz, 1H), 7.5 (bs, 1H), 7.1 (bs, 1H), 6.2 (dd, $J$ = 6.6, 7.7 Hz, 1H), 4.4 (s, 2H); $^{13}$C NMR (DMSO-$d_6$) $\delta$ 168.0, 154.1, 147.9, 139.5, 138., 103.3, 51.9

**2-(3-Amino-2-oxopyridin-1(2*H*)-yl)acetamide 20.** Compound **19** (0.55 g, 2.6 mmol) was suspended in MeOH (30 mL), and Pd/C (110 mg, 20% mass) was added. The reaction flask was flushed three times with hydrogen, and stirring was maintained under hydrogen atmosphere at room temperature for 4 h. The mixture was filtered through Celite, and the cake was washed thoroughly with MeOH (100 mL). The filtrate was evaporated to dryness to yield a crude brown powder, which was purified by flash chromatography on silica gel and eluted with $CH_2Cl_2$/MeOH/$Et_3N$, 95/5/1. The pure product was obtained as a beige powder: mp 208−210 °C (0.23 g, 53% yield); $^1$H NMR (DMSO-$d_6$) $\delta$ 7.5 (bs,1H), 7.1 (bs,1H), 6.8 (dd, $J$ = 1.7, 6.8 Hz, 1H), 6.4 (dd, $J$ = 1.7, 6.8 Hz, 1H), 6.0 (t, $J$ = 6.8 Hz, 1H), 5.0 (bs, 2H); $^{13}$C NMR (DMSO-$d_6$) $\delta$ 169.2, 157.5, 138.5, 126.1, 110.7, 105.9.

**[1-(1-Carbamoylmethyl-2-oxo-1,2-dihydropyridin-3-ylcarbamoyl)-2-methylpropyl]carbamic Acid Benzyl Ester 21.** To a solution of Z-V-OH (75 mg, 0.3 mmol) in dry DMF (6 mL) were added HOBt (40 mg, 0.3 mmol), EDCI (57 mg, 0.3 mmol), and *N*-methylmorpholine (33 $\mu$L, 0.3 mmol). The solution was stirred for 15 min, and then 2-(3-amino-2-oxopyridin-1(2*H*)-yl)acetamide **20** (50 mg, 0.3 mmol) was added. Stirring was maintained at room temperature for 48 h. The solvent was evaporated under reduced pressure, and the residue was purified by flash chromatography on silica gel, eluting with $CH_2Cl_2$/MeOH, 95/5. The pure product was obtained as a crystalline white solid: mp 217−219 °C (67 mg, 56% yield); $^1$H NMR (DMSO-$d_6$) $\delta$ 9.1 (s, 1H), 8.1 (dd, $J$ = 1.6 Hz, $J$ = 7.4 Hz, 1H), 7.7 (d, $J$ = 8.3 Hz, 1H), 7.5 (bs, 1H), 7.3−7.2 (m, 6H), 7.1 (bs, 1H), 6.2 (t, $J$ = 7.1 Hz, 1H), 4.9 (s, 2H), 4.5 (s, 2H), 4.0 (dd, $J$ = 6.8, 8.0 Hz, 1H), 2.1−1.9 (m, 1H), 0.8 (d, $J$ = 6.7 Hz, 6H); $^{13}$C NMR (DMSO-$d_6$) $\delta$ 171.3, 168.8, 157.1, 137.2, 128.7, 128.2, 128.1, 128.0, 127.8, 123.1, 122.3, 105.1, 65.9, 61.6, 51.6, 19.4, 18.3. Anal. ($C_{20}H_{24}N_4O_5$ ) C, H, N.

**2-Amino-*N*-(1-carbamoylmethyl-2-oxo-1,2-dihydropyridin-3-yl)-3-methylbutyramide 22.** To a solution of **21** (250 mg, 0.6 mmol) in MeOH (15 mL) was added Pd/C (75 mg, 30% mass). The reaction flask was flushed three times with hydrogen, and stirring was maintained under hydrogen atmosphere at room temperature for 20 h. The mixture was filtered through a mixture of Celite and silica, and the cake was washed thoroughly with MeOH (200 mL). The filtrate was evaporated to dryness to yield a white solid: mp 177−179 °C (110 mg, 66% yield); $^1$H NMR (DMSO-$d_6$) $\delta$ 10.4−10.3 (m, 1H), 8.4 (dd, $J$ = 1.8, 7.4 Hz, 1H), 7.7 (bs, 1H), 7.3 (dd, $J$ = 1.8, 6.8 Hz, 1H), 7.2 (bs, 1H), 6.3 (t, $J$ = 7.1 Hz, 1H), 4.6 (s, 2H), 4.1 (dd, $J$ = 4.9, 10.1 Hz, 1H), 3.2 (d, 2H, $J$ = 4.8 Hz), 2.3−2.2 (m, 1H), 0.9 (d, 6H, $J$ = 6.9 Hz); $^{13}$C NMR (DMSO-$d_6$) $\delta$ 174.3, 168.7, 157.1, 143.4, 121.4, 109.4, 105.1, 60.6, 51.6, 31.1, 19.7, 16.6. Anal. ($C_{12}H_{18}N_4O_3\cdot 0.7H_2O$) C, H, N

**2-Methoxy-5-nitrobenzoic Acid Hydrazide 25.** A solution of **24** (1 g, 3.2 mmol) in MeOH/HCl (3.5 N) was stirred for 24 h at room temperature. After evaporation of the solvent, the desired hydrochloride salt was obtained as a white powder: mp 201−203 °C (688 mg, 87% yield); $^1$H NMR (DMSO-$d_6$) $\delta$ 11.0 (s, 1H), 8.5 (m, 3H), 7.5 (d, $J$ = 7.5 Hz, 2H), 3.8 (s, 3H); $^{13}$C NMR (DMSO-$d_6$) $\delta$ 162.7, 162.3, 140.6, 129.3, 126.3, 121.0, 113.7, 57.6. Anal. ($C_8H_9N_3O_4\cdot$HCl) C, H, N.

**Synthesis of 26.** To a solution of acid **23** (100 mg, 0.15 mmol) and hydrazide **25** (37 mg, 0.15 mmol) in DMF (5 mL) under argon atmosphere was added $Et_3N$ (23 $\mu$L, 0.16 mmol). HOBt (11 mg, 0.086 mmol) and HBTU (62 mg, 0.16 mmol) in solution in DMF (1 mL) were then added, and the mixture was stirred at room temperature for 24 h. DMF was evaporated under reduced pressure, and the residue was dissolved in $CH_2Cl_2$ (50 mL). The reaction product precipitated when the organic phase was washed with 5% aqueous citric acid (10 mL). The crude solid was filtered and washed successively with 10% aqueous $Na_2CO_3$ (10 mL) and water (10 mL) to give an off-white powder: mp 159−161°C (90 mg, 70% yield); $^1$H NMR (DMSO-$d_6$) $\delta$ 8.8 (s, 1H), 8.1−8.3 (m, 3H), 7.9 (m, 3H), 7.8 (m, 2H), 7.0−7.2 (m, 2H), 6.9 (d, 1H), 4.5−4.0 (m, 7H), 4.0 (s, 3H), 3.6 (s, 3H), 2.8−2.7 (m, 4H), 2.5−2.1 (m, 6H), 1.6 (m, 1H), 1.5 (m, 2H), 0.9 (m, 18H); $^{13}$C NMR (DMSO-$d_6$) $\delta$ 173.0, 172.8, 171.1, 163.5, 160.0, 156.0, 139.0, 135.1, 128.0, 127.0, 126.1, 123.0, 120.8, 115.0, 114.0, 104.5, 65.5, 57.0, 56.5, 56.0, 51.0, 51.0, 40.0, 31.5, 24.5, 28.0, 23.0, 22.5, 19.5; ESI$^+$ MS $m/z$ 873.4 $(M + Na)^+$. Anal. ($C_{43}H_{58}N_6O_{12}\cdot 2H_2O$) C, H, N

**Synthesis of Molecular Tong 2.** To a solution of **26** (100 mg, 0.11 mmol) in MeOH (7 mL) was added Pd/C (30 mg, 30% mass). The reaction flask was flushed three times with hydrogen, and stirring was maintained under hydrogen atmosphere at room temperature for 20 h. The mixture was filtered through a mixture of Celite and silica, and the cake was washed thoroughly with MeOH (200 mL). The filtrate was evaporated to dryness to yield an off-white solid: mp 170−180 °C (85 mg, 88% yield); $^1$H NMR (DMSO-$d_6$) $\delta$ 8.8 (s, 1H), 8.3−8.1 (m, 3H), 7.9 (m, 3H), 7.8 (m,

2H), 7.2−7.0 (m, 2H), 6.9 (d, 1H), 4.5−4.0 (m, 7H), 3.9 (s, 3H), 3.6 (s, 3H), 2.8−2.7 (m, 4H), 2.5−2.1 (m, 6H), 1.6 (m, 1H), 1.5 (m, 2H), 0.9 (m, 18H); $^{13}$C NMR (DMSO-$d_6$) $\delta$ 173.0, 172.8, 171.5, 171.1, 163.5, 160.0, 156.0, 139.0, 135.1, 128.0, 127.0, 126.0, 123.0, 120.8, 115.0, 114.0, 104.5, 65.5, 57.0, 56.0, 51.0, 49.9, 39.0, 32.0, 29.5, 29.0, 23.8, 23.0, 23.0−22.0, 19.5; ESI$^+$ MS *m*/*z* 843.5 (M + Na)$^+$. Anal. ($C_{43}H_{60}N_6O_{10}\cdot 2H_2O$) C, H, N

**Synthesis of Molecular Tong 3.** To a solution of acid **23** (166 mg, 0.25 mmol) and amine **14** (100 mg, 0.25 mmol) in DMF (5 mL) under argon atmosphere was added $Et_3N$ (38 $\mu$L, 0.28 mmol). HBTU (106 mg, 0.28 mmol) in solution in DMF (1 mL) was then added, and the mixture was stirred at room temperature for 24 h. DMF was evaporated under reduced pressure, and the residue was dissolved in $CH_2Cl_2$ (50 mL). The reaction product precipitated when the organic phase was washed with 5% aqueous citric acid (10 mL). The crude solid was filtered and washed successively with 10% aqueous $Na_2CO_3$ (10 mL) and water (10 mL) to give a yellow powder: mp 160−172°C (86 mg, 35% yield); $^1$H NMR (DMSO-$d_6$) $\delta$ 8.4 (bs, 1H), 8.2 (m, 2H), 7.9 (m, 3H), 7.8 (m, 2H), 7.7 (m, 2H), 7.1−7.0 (m, 2H), 7.0−6.9 (2s, 2H), 6.1 (d, $J$ = 9.4 Hz, 1H), 4.9 (bs, 2H), 4.7 (s, 2H), 4.4−4.0 (m, 7H), 4.0 (m, 1H), 3.6 (s, 3H), 2.8−2.7 (m, 4H), 2.4−2.1 (m, 9H), 1.8−1.7 (m, 1H), 1.6−1.5 (m, 3H), 1.4−1.1 (m, 2H), 1.0 (d, $J$ = 3.0.4 Hz, 3H), 0.8 (m, 24H); $^{13}$C NMR (DMSO-$d_6$) $\delta$ 173.0, 172.8, 171.1, 170.8, 171.0, 167.0, 157.5, 156.0, 140.0, 135.0, 127.0, 125.0, 123.0, 115.0, 106.5, 104.5, 67.0, 65.5, 59.0, 58.0, 57.0, 56.0, 51.0, 49.9, 48.0, 39.0, 37.0, 32.0, 29.5, 29.0, 25.0, 23.8, 23.0, 21.0, 19.5, 17.0, 16.0, 12.0; ESI$^+$ MS *m*/*z* 1058.23 (M + Na)$^+$. Anal. ($C_{53}H_{78}N_8O_{13}\cdot 3H_2O$) C, H, N

**Synthesis of Molecular Tong 4.** To a solution of acid **23** (100 mg, 0.15 mmol) and amine **17** (60 mg, 0.15 mmol) in DMF (5 mL) under argon atmosphere was added $Et_3N$ (22 $\mu$L, 0.16 mmol). HBTU (113 mg, 0.30 mmol) in solution in DMF (1 mL) was then added, and the mixture was stirred at room temperature for 24 h. DMF was evaporated under reduced pressure, and the residue was dissolved in $CH_2Cl_2$ (50 mL). The organic phase was washed with 5% aqueous citric acid (10 mL), 10% aqueous $Na_2CO_3$ (10 mL), and water (10 mL). Trituration of the obtained yellow oil in $Et_2O$ (20 mL) gave a yellow powder: mp 138−144°C (55 mg, 34% yield); $^1$H NMR (DMSO-$d_6$) $\delta$ 8.1−7.9 (m, 5H), 7.7 (d, $J$ = 7.0 Hz, 2H), 7.2 (bs, 2H), 6.9 (d, $J$ = 7.0 Hz, 2H), 6.1 (bd, $J$ = 7.2 Hz, 2H), 4.7 (m, 4H), 4.4 (m, 1H), 4.2−4.1 (m, 2H), 4.1−4.0 (m, 4H), 3.6 (s, 3H), 2.4−2.2 (m, 4H), 2.2 (s, 6H), 2.2−1.9 (m, 6H), 1.7 (m, 1H), 1.4 (m, 2H), 1.4 (s, 9H), 0.9 (m, 18H); $^{13}$C NMR (DMSO-$d_6$) $\delta$ 172.9, 172.5, 171.7, 168.0, 158.0, 140.0, 129.0, 126.0, 123.0, 116.1, 106.1, 106.0, 80.0, 67.0, 58.0, 53.6, 51.5, 47.0, 41.5, 32.5, 31.0, 28.5, 25.6, 25.0, 20.0, 17.5; ESI$^+$ MS *m*/*z* 1064.22 (M + Na)$^+$. Anal. ($C_{55}H_{75}N_7O_{13}\cdot 3H_2O$) C, H, N

**Synthesis of Molecular Tong 5.** To a solution of acid **23** (50 mg, 0.076 mmol) and amine **22** (24 mg, 0.091 mmol) in DMF (5 mL) under argon atmosphere was added DIPEA (66 $\mu$L, 0.38 mmol). The mixture was stirred for 20 min at room temperature. HOBt (11 mg, 0.086 mmol) and HBTU (33 mg, 0.086 mmol) in solution in DMF (1 mL) were then added, and the mixture was stirred at room temperature for 48 h. DMF was evaporated under reduced pressure, and the residue was dissolved in $CH_2Cl_2$/MeOH, 90/10 (100 mL). The reaction product precipitated when the organic phase was washed with 5% aqueous citric acid (20 mL). The crude solid was filtered and washed successively with 10% aqueous $Na_2CO_3$ (10 mL) and water (10 mL) to give a white powder: mp 210−217 °C (48 mg, 70% yield); $^1$H NMR (DMSO-$d_6$) $\delta$ 9.1 (s, 1H), 8.2 (d, $J$ = 7.9 Hz, 1H), 8.1 (d, $J$ = 7.1 Hz, 1H), 8.0−7.9 (m, 3H), 7.7 (d, $J$ = 9.0 Hz, 2H), 7.6 (bs, 1H), 7.3 (d, $J$ = 6.7 Hz, 1H), 7.2 (bs, 1H), 7.1 (s, 2H), 6.9 (bd, $J$ = 9.0 Hz, 2H), 6.2 (t, $J$ = 7.1 Hz, 1H), 4.5 (s, 2H), 4.4−4.3 (m, 2H), 4.2−4.1 (m, 2H), 4.0 (m, 4H), 3.6 (s, 3H), 2.4−2.3 (m, 4H), 2.1−2.0 (m, 1H), 2.0−1.9 (m, 6H), 1.6 (m, 1H), 1.4 (t, $J$ = 4.8 Hz, 2H), 0.9−0.7 (m, 24 H); $^{13}$C NMR (DMSO-$d_6$) $\delta$ 172.8, 172.6, 172.0, 171.4, 171.2, 168.8, 157.5, 156.5, 136.2, 133.5, 129.4, 128.4, 124.2, 122.9, 116.4, 106.7, 105.1, 67.4, 59.5, 58.3, 57.7, 52.1, 51.7, 51.3, 41.2, 32.0, 30.6, 30.3, 30.1, 25.4, 24.5, 23.4, 22.1, 18.6, 18.5; ESI$^+$ MS *m*/*z* 928.6 (M + Na)$^+$. Anal. ($C_{47}H_{67}N_7O_{11}\cdot 1.5H_2O$ ) C, H, N

**Synthesis of Molecular Tong 6.** Acid **23** (96 mg, 0.15 mmol) and amine **11** (43 mg, 0.13 mmol) were dissolved in DMF (10 mL), and DIPEA (0.11 mL, 0.66 mmol) was added. The reaction mixture was stirred under argon atmosphere at room temperature for 20 min. HBTU (56 mg, 0.15 mmol) and HOBt (20 mg, 0.15 mmol) were added to the reaction flask, and the solution was stirred for 48 h at room temperature. DMF was evaporated under reduced pressure, and the residue was dissolved in $CH_2Cl_2$ (50 mL). The reaction product precipitated when the organic phase was washed with 5% aqueous citric acid (10 mL). The crude solid was filtered and washed successively with 10% aqueous $Na_2CO_3$ (10 mL) and water (10 mL) to give **6** as a white powder: mp >266 °C (58 mg, 50% yield); $^1$H NMR (DMSO-$d_6$) $\delta$ 10.4 (bs, 1H), 10.0 (bd, 2H), 8.1−8.0 (m, 5H), 7.8 (dd, $J$ = 8.9, 2.7 Hz, 1H), 7.8 (bd, 2H), 7.2−7.3 (m, 2H), 7.2 (d, $J$ = 9.1 Hz, 1H), 7.0−7.1 (m, 2H), 4.5−4.4 (m, 2H), 4.2−4.1 (m, 6H), 3.9 (s, 3H), 3.7 (s, 3H), 2.6−2.6 (m, 4H), 2.2−2.1 (m, 10H), 1.75−1.65 (m, 1H), 1.5 (t, $J$ = 7.2 Hz, 2H), 1.1−0.85 (m, 24 H); $^{13}$C NMR (DMSO-$d_6$) $\delta$ 172.7, 171.9, 171.8, 171.5, 170.6, 168.1, 157.1, 152.8, 136.0, 132.5, 129.3, 124.1, 123.8, 121.5, 116.3, 112.7, 106.3, 67.4, 60.1, 57.5, 56.6, 56.4, 51.8, 51.2, 41.2, 31.9, 30.7, 29.0, 24.4, 23.0, 22.2, 19.0, 18.7, 18.5; ESI$^+$ MS *m*/*z* 984.6 (M + Na)$^+$. Anal. ($C_{50}H_{71}N_7O_{12}\cdot 2.5H_2O$ ) C, H, N

**Enzymatic Studies.** The fluorogenic substrate DABCYL-$\gamma$-abu-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Gln-EDANS {DABCYL, 4-(4′-dimethylaminophenylazo)benzoyl; $\gamma$-abu, $\gamma$-amino butyric acid; EDANS, 5-[(2-aminoethyl)amino]naphthalene-1-sulfonic acid)} was purchased from Bachem (Germany). Other reagents and solvents were purchased from commercial sources. The fluorescence and absorbance measurements were performed using a Perkin-Elmer LS 50B spectrofluorometer and a Uvikon 941 spectrophotometer, respectively.

**Wild-Type and Mutated Proteases.** The four PR mutants, namely, D30N, I50V, V82A, and G48V/L90M, were constructed with the Quickchange site-directed mutagenesis kit (Stratagene) using the appropriate pairs of primers that confer the right substitution in the nucleotide sequence. The plasmid pET9PRWT bearing the wild-type sequence of the protease gene from the HIV (BRU) DNA clone (subclone of $\lambda$J19) was used as a template for the PCR amplification cycles. The resulting mutated plasmids were sequenced (Genome Express) on both coding and noncoding strands in order to check for the sole presence of the wanted mutation(s). WT PR and D30N, I50V, V82A, and G48V/L90M mutants were expressed in *E. coli* BL21(DE3) rosetta pLysS strain (Novagen) as inclusion bodies. Subsequent purification in denaturing conditions and refolding were performed according to Billich et al.[34] Plasmids encoding MDR-HM[31] and ANAM-11[32] mutants (generous gift of Prof. E. Freire, Johns Hopkins University, Baltimore, MD) were used to transform the *E. coli* BL21(DE3) rosetta pLysS strain, and after expression both mutants were purified according to Muzammil et al. [32]

**Enzyme and Inhibition Assays.** The enzymatic activities of wild-type and mutated proteases were determined fluorometrically using DABCYL-$\gamma$-abu-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Gln-EDANS ($\lambda_{ex}$ = 340 nm; $\lambda_{em}$ = 490 nm) in 100 mM sodium acetate, 1 mM EDTA, and 1 M NaCl at pH 4.7 and 30 °C.[23] Substrate and compounds were previously dissolved in DMSO, with the final solvent concentration kept constant at 3% (v/v). A typical inhibition experiment used 7.5 nM enzyme and 5.2 $\mu$M substrate. The $IC_{50}$ values (inhibitor concentration leading to 50% inhibition) were obtained by plotting the percent inhibition against inhibitor concentration (0.5−28 $\mu$M) and fitting the experimental data to

$$\%\ \text{inhibition} = 100 \times \frac{[I]_0}{[I]_0 + IC_{50}}$$

The mechanism of inhibition and the corresponding kinetic constants $K_{ic}$ (competitive inhibition) and $K_{id}$ (dimerization inhibition) were determined using Zhang−Poorman kinetic analysis.[16,22,23] Kinetics were carried out with a constant substrate concentration (5.2 $\mu$M) and at least five enzyme concentrations (4.7−43.5

nM). Inhibitor concentrations were 6 and 11 $\mu$M (compound **3**), 4 and 6 $\mu$M (compound **4**), 1 and 2 $\mu$M (compound **5**), and 0.6 and 1.2 $\mu$M (compound **6**). All experiments were performed in triplicate.

**Evaluation of Metabolic Stability.** The stability of inhibitors in RPMI culture medium containing 20% fetal calf serum was assessed by studying their kinetics of breakdown at 37 °C for up to 2 days. Incubation was terminated by adding ethanol. The mixture was poured at 4 °C and centrifuged (10 000 rpm for 10 min). Aliquots (20 $\mu$L) of the clear supernatant were injected onto the RP-HPLC column (Waters 996). The degradation half-life was obtained by a least-squares linear regression analysis of a plot of the log[I] vs time using a minimum of five points.

**Acknowledgment.** The authors thank Prof. Ernesto Freire of the Department of Biology and Biocalorimetry Center, The Johns Hopkins University, Baltimore, MD, for his generous gift of plasmids encoding MDR-HM and ANAM-11 mutants. L.B. holds a doctoral fellowship from MNSER. The English text was edited by Dr. Owen Parkes.

**Supporting Information Available:** Results from elemental analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

## References

(1) Kohl, N. E.; Emini, E. A.; Schleif, W. A.; Davis, L. J.; Heimbach, J. C.; Dixon, R. A. F.; Scolnick, E. M.; Sigal, I. S. Active Human Immunodeficiency Virus Protease Is Required for Viral Infectivity. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 4686−4690.

(2) Peng, C.; Ho, B. K.; Chang, T. W.; Chang, N. T. Role of Human Immunodeficiency Virus Type 1-Specific Protease in Core Protein Maturation and Viral Infectivity. *J. Virol.* **1989**, *63*, 2550−2556.

(3) Tomasselli, A. G.; Heinrickson, R. L. Targeting the HIV-Protease in AIDS Therapy: A Current Clinical Perspective. *Biochim. Biophys. Acta* **2000**, *1477*, 189−214.

(4) De Clercq, E. New Anti-HIV Agents and Targets. *Med. Res. Rev.* **2002**, *22*, 531−565.

(5) Golsmith, D. R.; Perry, C. M. Atazanavir. *Drugs* **2003**, *63*, 1679−1693.

(6) Gulick, R M. New Antiretroviral Drugs. *Clin. Microbiol. Infect.* **2003**, 186−193.

(7) D'Aquila, R. T.; Schapiro, J. M.; Brun-Vezinet, F.; Clotet, B.; Conway, B.; Demeter, L. M.; Grant, R. M.; Johnson, V. A.; Kuritzkes, D. R.; Loveday, C.; Shafer, R. W.; Richman, D. D. Drug Resistance Mutations in HIV-1. *Top. HIV Med.* **2002**, *10*, 21−25.

(8) Larder, B. Mechanisms of HIV-1 Drug Resistance. *AIDS* **2001**, *15* (Suppl. 5), S27−S34.

(9) Ishima, R.; Ghirlando, R.; Tozser, J.; Gronenborn, A. M.; Torchia, D. A.; Louis, J. M. Folded Monomer of HIV-1 Protease. *J. Biol. Chem.* **2001**, *276*, 49110−49116.

(10) Todd, M. J.; Semo, M.; Freire, E. The Structural Stability of HIV-1 Protease. *J. Mol. Biol.* **1998**, *283*, 475−488.

(11) Schinazi, R. F.; Larder, B. A.; Mellors, J. W. Mutations in Retroviral Genes Associated with Drug-Resistance. *Antiviral News* **1997**, *5*, 129−142.

(12) Kuiken, C. L.; Foley, B.; Hahn, B.; Korber, B.; Marx, P. A.; McCutchan, F.; Mellors, J. W.; Wolinksy, S. Technical Report LA-UR02−2877; Los Alamos National Laboratory: Los Alamos, NM, 2001.

(13) Gustchina, A.; Weber, I. T. Comparative Analysis of the Sequences and Structures of HIV-1 and HIV-2 Proteases. *Proteins* **1991**, *10*, 325−329.

(14) Boggetto, N.; Reboud-Ravaux, M. Dimerization Inhibitors of HIV-1 Protease. *Biol. Chem.* **2002**, *383*, 1321−1324.

(15) Schramm, H. J.; Nakashima, H.; Schramm, W.; Wakayama, H.; Yamamoto, N. HIV-1 Reproduction Is Inhibited by Peptides Derived from the N- and C-Termini of HIV-1 Protease. *Biochem. Biophys. Res. Commun.* **1991**, *179*, 847−851.

(16) Zhang, Z.-Y.; Poorman, R. A.; Maggiora, L. L.; Heinrickson, R. L.; Kedzy, F. J. Dissociative Inhibition of Dimeric Enzymes. Kinetic Characterization of the Inhibition of HIV-1 Protease by Its COOH-Terminal Tetrapeptide. *J. Biol. Chem.* **1991**, *266*, 15591−15594.

(17) Franciskovich, J.; Houseman, K.; Mueller, R.; Chmielewski, J. A Systematic Evaluation of the Inhibition of HIV-1 Protease by Its C- and N-Terminal Peptides. *Bioorg. Med. Chem. Lett.* **1993**, *3*, 765−768.

(18) Schramm, H. J.; de Rosny, E.; Reboud-Ravaux, M.; Büttner, J.; Dick, A.; Schramm, W. Lipopeptides as Dimerization Inhibitors of HIV-1 Protease. *Biol. Chem.* **1999**, *380*, 593−596.

(19) Dumond, J.; Boggetto, N.; Schramm, H. J.; Schramm, W.; Takahashi, M.; Reboud-Ravaux, M. Thyroxine-derivatives of lipopeptides: bifunctional dimerization inhibitors of immunodeficiency virus-1 protease. *Biochem. Pharmacol.* **2002**, *7550*, 1−6.

(20) Zutshi, R.; Franciskovich, J.; Slultz, M.; Schweitzer, B.; Bishop, P.; Wilson, M.; Chmielewski, J. Targeting the Dimerization Interface of HIV-1 Protease: Inhibition with Cross-Linked Interfacial Peptides. *J. Am. Chem. Soc.* **1997**, *119*, 4841−4845.

(21) Ulysse, L. G.; Chmielewski, J. Restricting the Flexibility of Crosslinked, Interfacial Peptide Inhibitors of HIV-1 Protease. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 3281−3286.

(22) Bouras, A.; Boggetto, N.; Benatalah, Z.; de Rosny, E.; Sicsic, S.; Reboux-Ravaud, M. Design, Synthesis, and Evaluation of Conformationally Constrained Tongs, New Inhibitors of HIV-1 Protease Dimerization. *J. Med. Chem.* **1999**, *42*, 957−962.

(23) Merabet, N.; Dumond, J.; Collinet, B.; Van Baelinghem, L.; Boggetto, N.; Ongeri, S.; Ressad, F.; Reboud-Ravaux M.; Sicsic, S. New Constrained "Molecular Tongs" Designed To Dissociate HIV-1 Protease Dimer. *J. Med. Chem.* **2004**, *47*, 6392−6400.

(24) Breccia, P.; Boggetto, N.; Pérez-Fernández, R.; Van Gool, M.; Takahashi, M.; René, L.; Prados, P.; Badet, B.; Reboud-Ravaux, M.; de Mendoza, J. Dimerization Inhibitors of HIV-1 Protease Based on a Bicyclic Guanidinium Subunit. *J. Med. Chem.* **2003**, *46*, 5196−5207.

(25) (a) Nowick, J. S.; Holmes, D. L.; Mackin, G.; Noronha, G.; Shaka, A. J.; Smith, E. M. An Artificial $\beta$-Sheet Comprising a Molecular Scaffold, a $\beta$-Strand Mimic, and a Peptide Strand. *J. Am. Chem. Soc.* **1996**, *118*, 2764−2765. (b) Nowick, J. S.; Pairish, M.; Lee, I. Q.; Holmes, D. L.; Ziller, J. W. An Extended $\beta$-Strand Mimic for a Larger Artificial $\beta$-Sheet *J. Am. Chem. Soc.* **1997**, *119*, 5413−5424. (c) Nowick, J. S.; Chung, D. M.; Maitra, K.; Stigers, K. D.; Sun, Y. An Unnatural Amino Acid That Mimics a Tripeptide $\beta$-Strand and Forms $\beta$-Sheetlike Hydrogen-Bonded Dimers. *J. Am. Chem. Soc.* **2000**, *122*, 7654−7661. (d) Nowick, J. S.; Cary, J. M.; Tsai, J. H. A Triply Templated Artificial $\beta$-Sheet *J. Am. Chem. Soc.* **2001**, *123*, 5176−5180. (e) Tsai, J. H.; Nowick, J. S. Two New $\beta$-Strand Mimics *Bioorg. Med. Chem.* **1999**, *7*, 29−38. (f) Nowick J. S.; Smith, E. M.; Ziller, J. W.; Shaka, A. J. Three-Stranded Mixed Artificial $\beta$-Sheets. *Tetrahedron* **2002**, *58*, 727−739.

(26) Sanderson, P. E. J.; Lyle, T. A.; Cutrona, K. J.; Dyer, D. L.; Dorsey, B. D.; McDonough, C. M.; Naylor-Olsen, A. M.; Chen, I.-W.; Chen, Z.; Cook, J. J.; Cooper, C. M.; Gardell, S. J.; Hare, T. R.; Krueger, J. A.; Lewis, S. D.; Lin, J. H.; Lucas, B. J., Jr.; Lyle, E. A.; Lynch, J. J., Jr.; Stranieri, M. T.; Vastag, K.; Yan, Y.; Shafer, J. A.; Vacca, J. P. Efficacious, Orally Bioavailable Thrombin Inhibitors Based on 3-Aminopyridinone or 3-Aminopyrazinone Acetamide Peptidomimetic Templates. *J. Med. Chem.* **1998**, *41*, 4466−4474.

(27) Clemente, J. C.; Hemrajani, R.; Blum, L. E.; Goodenow, M. M.; Dunn, B. M. Secondary Mutations M36I and A71V in the Human Immuodeficiency Virus Type 1 Protease Can Provide an Advantage for the Emergence of the Primary Mutation D30N. *Biochemistry* **2003**, 15029−15035.

(28) Ahlsen, G.; Hulten, J.; Poliakov, A.; Lindgren, M. T.; Alterman, M.; Samuelsson, B.; Hallberg, A.; Danielson, U. H. Resistance Profile of Cyclic and Linear Inhibitors of HIV-1 Protease. *Antiviral Chem. Chemother.* **2002**, *13*, 27−37.

(29) Pazhanisamy, S.; Stuver, C. M.; Cullinan, A. B.; Margolin, N.; Rao, B. G.; Livingston, D. Kinetic Characterization of Human Immunodeficiency Virus Type-1 Protease-Resistant Variants. *J. Biol. Chem.* **1996**, 17979−17985.

(30) Maschera, B.; Darby, G.; Palu, G.; Wright, L. L.; Tisdale, M.; Myers, R.; Blair, E. D.; Furfine, E. S. Mutations in the Viral Protease That Confer Resistance to Saquinavir Increase the Dissociation Rate Constant of the Protease−Saquinavir Complex. *J. Biol. Chem.* **1996**, *271*, 33231−33235.

(31) Ohtaka, H.; Schön, A.; Freire, E. Multidrug Resistance to HIV-1 Protease Inhibition Requires Cooperative Coupling between Distal Mutations. *Biochemistry* **2003**, *42*, 13659−13666.

(32) Muzammil, S.; Ross, P.; Freire, E. A Major Role for a Set of Non-Active Site Mutations in the Development of HIV-1 Protease Drug Resistance. *Biochemistry* **2003**, *42*, 631−638.

(33) Shulz, M. D.; Ham, Y.-U.; Lee, S.-G.; Davis, D. A.; Brown, C.; Chmielewski, J. Small-Molecule Dimerization Inhibitors of Wild-Type and Mutant HIV Protease: A Focused Library Approach. *J. Am. Chem. Soc.* **2004**, *126*, 9886−9887.

(34) Billich, A.; Hammerschmid, F.; Winkler, G. Purification Assay and Kinetic Features of HIV-1 Proteinase. *Biol. Chem. Hoppe−Seyler* **1990**, *371*, 265−272.

JM060576K