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Crucial amides for dimerization inhibitors of HIV-1 protease

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Abstract—An inhibitor based on crosslinked peptides from the interfacial region of HIV-1 protease, previously shown to act by dimerization inhibition, was modified by *N*-methylation to ascertain the importance of the amide hydrogens on inhibition. The effects of *N*-methylation on HIV-1 protease inhibition, as well as the effects on degradation by proteases are described.

HIV-1 protease (HIV-1 PR) exists as a homodimer[1] where half of the active site, residues Asp25-Thr26-Gly27, are found within each monomer.[2] The primary function of HIV PR is site specific cleavage of the polypeptide products of the viral gag and gag-pol genes;[3] without proper processing the virion remains in an immature form.[4] Virions arising from transfection with a provirus mutated in the protease-coding region contained unprocessed viral proteins and were non-infectious.[5] Current FDA approved agents combat HIV infection through inhibition of the active site of HIV PR. Although these inhibitors have been shown to be potent in vivo, the effect is often short-lived as strains containing mutations around the active site become more prevalent.[6]

One potential alternative mode of inhibition would be to target the dimerization interface of HIV PR. Dimerization is essential for catalytic activity as the substrate binding site and active site are formed from interactions of each monomer.[7] A major portion of the stabilizing interactions for dimerization is generated by an interdigitated four-stranded β-sheet structure (Fig. 1),[8] containing the C- and N-terminal residues of HIV-1 PR. The dimerization interface has been identified to be highly conserved among HIV-1 isolates,[9] as a mutation in any position (except Leu97) would, most likely, require a complementary mutation on the opposite terminus of the enzyme to confer stabilization to the dimeric structure.

In order to mimic the native structure of the conserved β-sheet region of HIV-1 PR, a non-peptide based linker has been used to connect the two N-termini of the interfacial peptides, leading to a drastic 500-fold increase in inhibitory activity over the peptides alone.[10] Truncation of the interdigitating peptides and modification of the tryptophan residue provided a lower molecular weight inhibitor, **1**, shown to maintain the dissociative mechanism.[11] The demonstration that a one residue mutation could lead to an increase in inhibition was also shown.[11] These findings indicated that the side-chain interactions may be more important to inhibition than the β-sheet forming amide backbone.

With this in mind, as well as the desire to reduce the number of amide linkages to lower the potential for proteolysis, the synthesis and testing of *N*-methylated variants (**2**–**6**) of **1** were undertaken to determine the contribution of each amide N–H functionality to inhibition

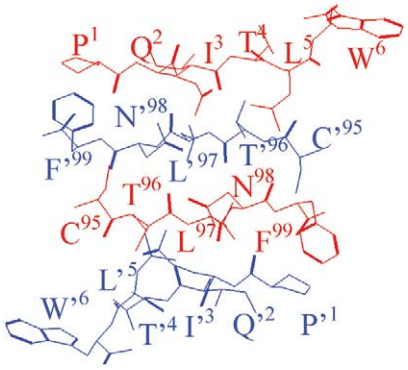

Figure 1. The four-stranded interdigitated β-sheet dimerization interface of HIV-1 protease. Monomers are colored in blue and red.

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(Fig. 2). Rather than modifying the terminal carboxamide the choice was made to eliminate it from the structure (**7**) (Fig. 2), as our model suggests that the carboxamide is not involved in hydrogen bonding. We expected that removal of amide hydrogens involved in the hydrogen bonding network of the β-sheet would lead to a 10–20-fold (≈1–2.5 kcal/mol) loss in activity when replaced with a methyl group due to the loss of one hydrogen bond.

The designed agents incorporating *N*-methyl groups could be synthesized from fragments according to the retrosynthesis shown in Scheme 1. Compounds **1**–**4** were synthesized from the reaction of **14**, **15**, and the appropriately modified resin bound tripeptide (**8**–**11**)

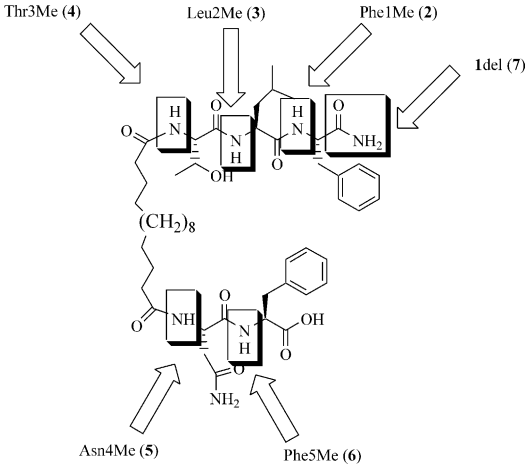
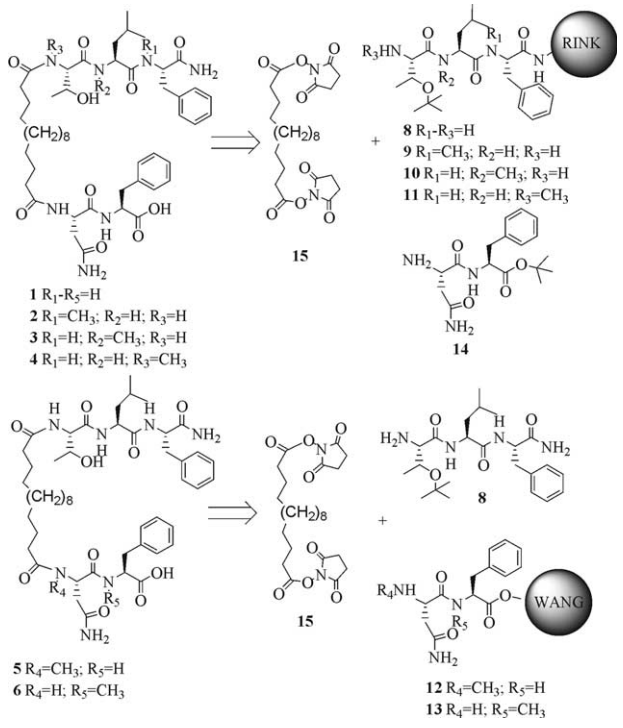


Figure 2. Depiction of a methylated library of analogues of **1** where arrows indicate individual points of methylation and the deleted carboxamide of **7**.



Scheme 1. Retrosynthesis of compounds **1**–**6**.

(Scheme 1). Compounds **5** and **6** were synthesized from the reaction of **8**, **15**, and appropriately modified dipeptides **12** and **13**. Variants **2**, **3** and **6** were synthesized using commercially available Fmoc- protected *N*-methyl amino acids on the solid support using Rink resin[12] or Wang resin[13] (**6**). HATU was employed as the coupling reagent to activate the Fmoc protected amino acid reacting with the secondary amine, as it has been shown to provide superior yields and reduced racemization as compared to the use of HBTU for reactions with hindered and methylated amines.[14] The synthesis of compounds **2** and **3** was completed when the resin bound tripeptides (**8**–**10**) were reacted with **15** and **14**, followed by cleavage from the solid support.[15]

Methylated threonine was prohibitively expensive and methylated asparagine was not commercially available, therefore, compounds **11** (on Rink resin) and **12** (on Wang resin) were synthesized on the resin using a site specific methylation methodology developed by Scanlan et al.[16] This procedure worked well in the synthesis of **4** and **5**, followed by cleavage from the solid support.[17]

Compound **13** was synthesized on the Wang resin using standard Fmoc-based protocols for the addition of Fmoc-asparagine to commercially available resin bound *N*-methylated phenylalanine. However, the completion of the synthesis of **6** did not occur using resin bound **13**. Under the reaction conditions between resin bound dipeptide (**13**) with **15** and **8** at 60 °C, **13** cyclized forming the diketopiprazine. This was due, presumably, to the lowered barrier for *cis-trans* isomerization of the methylated amide allowing for an orientation favorable for cyclization. To circumvent this problem the Fmoc protected *N*-methylated dipeptide was cleaved from the resin, the Fmoc protecting group removed, and the resulting dipeptide was purified by RP-HPLC. The resin free compound **13** was then reacted in solution with **15** and **8** to provide **6**.[18]

Compound **7** lacking the N-terminal carboxamide could not be synthesized on the resin due to the lack of the normal point of attachment for solid phase peptide synthesis; therefore, it was synthesized in solution from the components, utilizing phenethylamine in place of phenylalanine.[19]

Evaluation of inhibitory activity against HIV-1 PR of compounds **2**–**7** was performed using the assay developed by Toth and Marshall.[20] The IC_{50} values obtained showed that the *N*-hydrogens of the amides do not appear to be extremely important for inhibition with differences ranging from 0–2-fold decreases in potency (Table 1). Interestingly **7** did not lose any potency after removal of the carboxamide as compared to **1.** These data indicate that the hydrogen bonds in the dimerization interface of HIV-1 protease may not be as important as the side chain interactions. Rich et al. have reported that a subunit from the natural product Didemnaketal A can inhibit HIV-1 PR dimerization despite the subunits containing no amide bonds,[21] providing further support that amide bonds may not be necessary for dimerization inhibition.

Table 1. Inhibition of HIV-1 protease with non-methylated (**1**), methylated (**2**–**6**), and amide deletion (**7**) compounds

Compd	IC_{50} μM[a,b]	Relative loss of potency
1	5.9 (±0.6)	1.0
2	13 (±1)	2.2
3	8.0 (±0.8)	1.4
4	8.3 (±0.9)	1.4
5	4.5 (±0.9)	—
6	9.6 (±0.3)	1.6
7	5.8 (±0.5)	—

[a] Errors are given in parentheses.
[b] HIV-1 protease concentration was 25 nM.

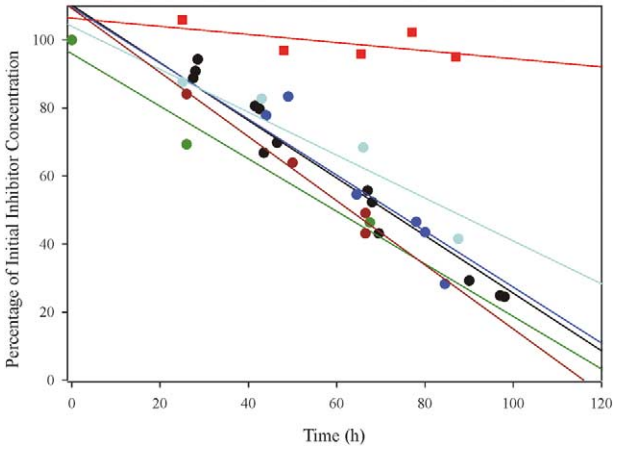


Figure 3. Pepsin digest of methylated and non-methylated inhibitors as monitored by HPLC expressed as a ratio compared to initial inhibitor concentration. ● Compound **1**; ● Compound **2** (Phe1Me); ■ Compound **3** (Leu2Me); ● Compound **4** (Thr3Me); ● Compound **5** (Asn4Me); ● Compound **6** (Phe5Me). Inhibitors (160 μM) were incubated with pepsin (buffer: 1.5 M AcOH, pH 2.1) or pronase (100 mM Tris, 5 mM $CaCl_2$, pH 8) at 37 °C for the designated period of time.

A potential positive outcome of *N*-methylation would be a reduced susceptibility to proteases in vivo. To test whether **2**–**6** were more stable to proteolysis than their non-methylated counterpart **1**, the methylated compounds were treated with the proteases pepsin and pronase. All compounds (**1**–**6**) were degraded by the non-specific protease pronase within 48 h. Compounds **1**, **2**, **4**–**6** were significantly degraded by the protease pepsin within 72 h, however, compound **3** was not proteolyzed by pepsin after 96 h (Fig. 3). This demonstrates that methylation of specific amides can reduce the proteolysis of inhibitors potentially increasing bioavailability without severely compromising the activity.

An exciting result of this work is the potential to use an organic scaffold in place of the peptide linkages to display the side chains in an appropriate geometry to inhibit the dimerization of HIV-1 PR. The use of alternate structures to displaying the desired functionality can be more robust for use in biological systems than the amide linkages of the peptides. Additionally, the use of phenethylamine derivatives in place of a phenylalanine in the northern tripeptide can allow for various functionalities to be incorporated on the phenyl ring without the use of unnatural amino acids.

Acknowledgements

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15. Compound **1**: RP-HPLC: 35% Acetonitrile/0.1% TFA/65% H_2O/0.1% TFA to 70% Acetonitrile/0.1% TFA/30% H_2O/0.1% TFA. Retention time: 33.9 min. Electrospray Ionization Mass Spectrometry: calculated, 907; found: 908.3 $(M+H^+)$; 931.2 $(M+Na^+)$; 946.4 $(M+K^+)$. Amino acid analysis: Asx 0.9 (1.0), Thr 0.8 (1.0), Leu 1.0 (1.0), Phe 2.0 (2). ^1H NMR (300 MHz) in DMSO-d_6, 0.84 (m, 6H), 1.00 (d (J=6.21 Hz) 3H), 1.25 (bs, 20H), 1.41 (m, 6H), 1.56 (bm, 1H), 2.04 (m, 4H), 2.45 (m, 2H), 2.95 (m, 4H), 3.93 (bm, 1H), 4.25 (d, J=7.6 Hz, 1H), 4.39 (m, 1H), 4.45 (m, 2H), 4.58 (m, 1H), 6.91 (s, 1H), 7.23 (s, 1H), 7.21 (bm, 10H), 7.69 (d (J=8.02 Hz), 1H), 7.80 (d (J=7.68 Hz), 1H), 7.86 (d (J=8.44 Hz), 1H), 7.97 (d (J=8.14 Hz), 1H), 8.08 (d (J=7.88 Hz), 1H).
Compound **2**: RP-HPLC: 30% Acetonitrile/0.1% TFA/70% H_2O/0.1% TFA to 70% Acetonitrile/0.1% TFA/30% H_2O/0.1% TFA. Retention time: 42.4 min. Matrix assisted Laser Desorption Mass Spectrometry: calculated, 921.5; found: 947 $(M+Na^+)$; 963 $(M+K^+)$. Amino acid analysis: Asx 1.0 (1.0), Thr 1.0 (0.9), Leu 1.0 (1.0), Phe 1.0 (0.8). ^1H NMR (300 MHz) in DMSO-d_6: 0.84 (m, 6H),

1.00 (d (J = 6.21 Hz) 3H), 1.25 (bs, 20H), 1.41 (m, 6H), 1.56 (bm, 1H), 2.04 (m, 4H), 2.45 (m, 2H), 2.75 (s, 3H), 2.95 (m, 4H), 3.93 (bm, 1H), 4.25 (d, J = 7.6 Hz, 1H), 4.30–4.40 (m, 2H), 4.45 (m, 1H), 4.58 (m, 1H), 6.91 (s, 1H), 7.23 (s, 1H), 7.21 (bm, 10H), 7.69 (d (J = 8.02 Hz), 1H), 7.86 (d (J = 8.44 Hz), 1H), 7.97 (d (J = 8.14 Hz), 1H), 8.08 (d, (J = 7.88 Hz), 1H). Compound **3**: RP-HPLC: 30% Acetonitrile/0.1% TFA/70% H_2O/0.1% TFA to 70% Acetonitrile/0.1% TFA/30% H_2O/0.1% TFA. Retention time: 38.5 min. Matrix assisted Laser Desorption Mass Spectrometry: calculated 921.5; found 945.8 ($M + Na^+$); 967.9 ($M + K^+$). Amino acid analysis: Asx 1.0 (1.0), Thr 1.0 (0.8), Leu 0.0 (0.0), Phe 2.0 (1.8). ^{1}H NMR (300 MHz) in DMSO-d_6: 0.84 (m, 6H), 1.00 (d (J = 6.21 Hz) 3H), 1.25 (bs, 20H), 1.41 (m, 6H), 1.56 (bm, 1H), 2.04 (m, 4H), 2.21 (s, 3H), 2.45 (m, 2H), 2.95 (m, 4H), 3.93 (bm, 1H), 4.25 (d, J = 7.6 Hz, 1H), 4.35 (m, 1H), 4.45 (m, 2H), 4.58 (m, 1H), 6.91 (s, 1H), 7.23 (s, 1H), 7.21 (bm, 10H), 7.69 (d (J = 8.02 Hz), 1H), 7.80 (d (J = 7.68 Hz), 1H), 7.86 (d (J = 8.44 Hz), 1H), 8.08 (d, (J = 7.88 Hz), 1H).

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17. Compound **4**: RP-HPLC: 35% Acetonitrile/0.1% TFA/65% H_2O/0.1% TFA to 65% Acetonitrile/0.1% TFA/35% H_2O/0.1% TFA. Retention time: 36.6 min. Electrospray Ionization Mass Spectrometry: calculated, 921.5; found 946 ($M + Na^+$). Amino acid analysis: Asx 1.0 (1), Thr 0.0 (0), Leu 1.3 (1), Phe 2.0 (2). ^{1}H NMR (300 MHz) in DMSO-d_6: 0.84 (m, 6H), 1.00 (d (J = 6.21 Hz) 3H), 1.25 (bs, 20H), 1.41 (m, 6H), 1.56 (bm, 1H), 2.04 (m, 4H), 2.45 (m, 2H), 2.65 (s, 3H), 2.95 (m, 4H), 3.93 (bm, 1H), 4.15 (d (J = 7.6 Hz), 1H), 4.39 (m, 1H), 4.45 (m, 2H), 4.58 (m, 1H), 6.91 (s, 1H), 7.23 (s, 1H), 7.21 (bm, 10H), 7.80 (d (J = 7.68 Hz), 1H), 7.86 (d (J = 8.44 Hz), 1H), 7.97 (d (J = 8.14 Hz), 1H), 8.08 (d (J = 7.88 Hz), 1H). Compound **5**: RP-HPLC: 35% Acetonitrile/0.1% TFA/65% H_2O/0.1% TFA to 65% Acetonitrile/0.1% TFA/35% H_2O/0.1% TFA. Retention time: 36.6 min. Electrospray Ionization Mass Spectrometry: calculated, 923; found: 946 ($M + Na^+$). Amino acid analysis: Asx 0.0 (0), Thr 0.9 (1), Leu 1.0 (1), Phe 1.8 (2). ^{1}H NMR (300 MHz) in DMSO-d_6: 0.84 (m, 6H), 1.00 (d (J = 6.21 Hz) 3H), 1.25 (bs, 20H), 1.41 (m, 6H), 1.56 (bm, 1H), 2.04 (m, 4H), 2.45 (m, 2H), 2.85–3.05 (m, 7H, including s, 3H), 3.93 (bm, 1H), 4.25 (d (J = 7.6 Hz), 1H), 4.39 (m, 1H), 4.45–4.60 (m, 3H), 6.91 (s, 1H), 7.23 (s, 1H), 7.21 (bm, 10H), 7.69 (d (J = 8.02 Hz), 1H), 7.80 (d (J = 7.68 Hz), 1H), 7.86 (d (J = 8.44 Hz), 1H), 7.97 (d (J = 8.14 Hz), 1H).
18. Compound **6**: RP-HPLC: 60 min. Linear gradient of 30% Acetonitrile/0.1% TFA /70% H_2O/0.1% TFA to 60% Acetonitrile/0.1% TFA/40% H_2O/0.1% TFA. Retention time: 43.4 min. Electrospray Ionization Mass Spectrometry: calculated, 923; found: 946 ($M + Na^+$). Amino acid analysis: Asx 1.0 (1), Thr 0.9 (1), Leu 1.0 (1), Phe 0.9 (1). ^{1}H NMR (300 MHz) in DMSO-d_6, 0.84 (m, 6H), 1.00 (d (J = 6.21 Hz) 3H), 1.25 (bs, 20H), 1.41 (m, 6H), 1.56 (bm, 1H), 2.04 (m, 4H), 2.45 (m, 2H), 2.78 (s, 3H), 2.95 (m, 4H), 3.93 (bm, 1H), 4.25 (d (J = 7.6 Hz), 1H), 4.25–4.39 (m, 2H), 4.45 (m, 1H), 4.58 (m, 1H), 6.91 (s, 1H), 7.23 (s, 1H), 7.21 (bm, 10H), 7.69 (d (J = 8.02 Hz), 1H), 7.80 (d (J = 7.68 Hz), 1H), 7.97 (d (J = 8.14 Hz), 1H), 8.08 (d (J = 7.88 Hz), 1H).
19. Compound **7**: RP-HPLC: 60 min. Linear gradient of 30% Acetonitrile/0.1% TFA/70% H_2O/0.1% TFA to 95% Acetonitrile/0.1% TFA/5% H_2O/0.1% TFA. Retention time: 41.2 min. Matrix Assisted Laser Desorption Mass Spectrometry: calculated, 865; found: 865.7 ($M + H^+$). Amino acid analysis: Asx 1.0 (1), Thr 0.8 (1), Leu 1.0 (1), Phe 1.0 (1). ^{1}H NMR (300 MHz) in DMSO-d_6, 0.84 (m, 6H), 1.00 (d (J = 6.21 Hz) 3H), 1.25 (bs, 20H), 1.41 (m, 6H), 1.56 (bm, 1H), 2.04 (m, 4H), 2.19 (t (7.0 Hz), 2H), 2.45 (m, 2H), 2.66 (t (7.2 Hz), 2H), 2.95 (m, 2H), 3.93 (bm, 1H), 4.25 (d (J = 7.6 Hz), 1H), 4.39 (m, 1H), 4.45 (m, 1H), 4.58 (m, 1H), 6.90–7.10 (m, 6H), 7.23 (s, 1H), 7.21 (bm, 5H), 7.69 (d (J = 8.02 Hz), 1H), 7.80 (d (J = 7.68 Hz), 1H),7.97 (d (8.14 Hz), 1H), 8.08 (d (J = 7.88 Hz), 1H).
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