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Hydrophobicity versus activity in crosslinked interfacial peptide inhibitors of HIV-1 protease †

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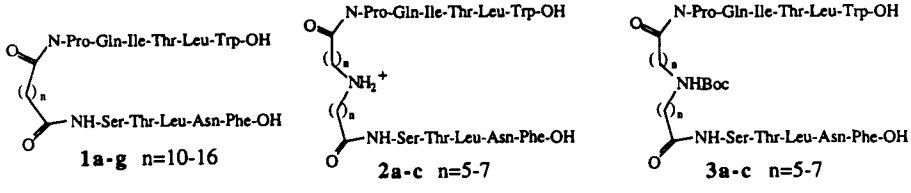
Abstract: Crosslinked peptides with amino functionality incorporated within the tethering moiety **2a–c**, **3a–c** were synthesized and tested as interfacial inhibitors of HIV-1 protease. As part of a strategy to develop the novel method of dissociative inhibition as a viable pharmacological approach, compounds were made to test hydrophobicity and size requirements of the tethering moiety. In the case of crosslinked interfacial peptide inhibitors of HIV-1 protease, hydrophilic **2a–c** and bulky hydrophobic **3a–c** tethers decreased effectiveness by approximately 10- and 2-fold, respectively. © 1997 Published by Elsevier Science Ltd. All rights reserved.

HIV-1 protease (PR), a dimeric enzyme composed of 99 amino acids, is responsible for processing the gag and gag/pol polypeptide precursors of HIV.[1] PR is essential for viral replication and as such, the enzyme is a significant target for inhibition.[2] Although most inhibitors have been targeted to the active site, inhibition of dimerization has recently been shown to be a viable means of PR inhibition. The dimeric interface of PR is composed of a four stranded β-sheet containing the N- and C-terminal regions of the protein.[3] Short peptides corresponding to the N- and C-termini of PR have shown weak, but specific, inhibition of dimerization.[4] Crosslinking these interfacial peptides via polymethylene tethers **1a–g**, significantly increased inhibition of PR as compared to the peptides alone or in combination.[5] In the crystal structure of PR, however, the terminal amine of Pro (1) interacts with the C-terminus Phe (99) potentially via electrostatic interactions.[3] One potential drawback of linking the interfacial peptides via carboxamides, as in **1a–g**, is the removal of this salt bridge, leading potentially to reduced potency. Amine based tethers of variable length **2a–c** were investigated, therefore, to probe the effectiveness of regenerating this ion pair in interfacial PR inhibitors.



Design of inhibitors

An idealized representation of the designed inhibitor/protease complex demonstrated that a basic functionality incorporated into the middle of a variable length tether **2a–c**, might improve inhibition by increasing electrostatic interactions between the Phe (99) carboxylate and the protonated amine of the tether (Figure 1). Amino-functionality, therefore, was incorporated into the middle of tethering moieties. These compounds are comparable in tether length to **1b**, **1d** and **1f**, so direct comparison between compounds **1** and **2** could be used to assess the role of the amino-group in PR inhibition. A

† Dedicated to Professor Herbert C. Brown on the occasion of his 85th birthday.

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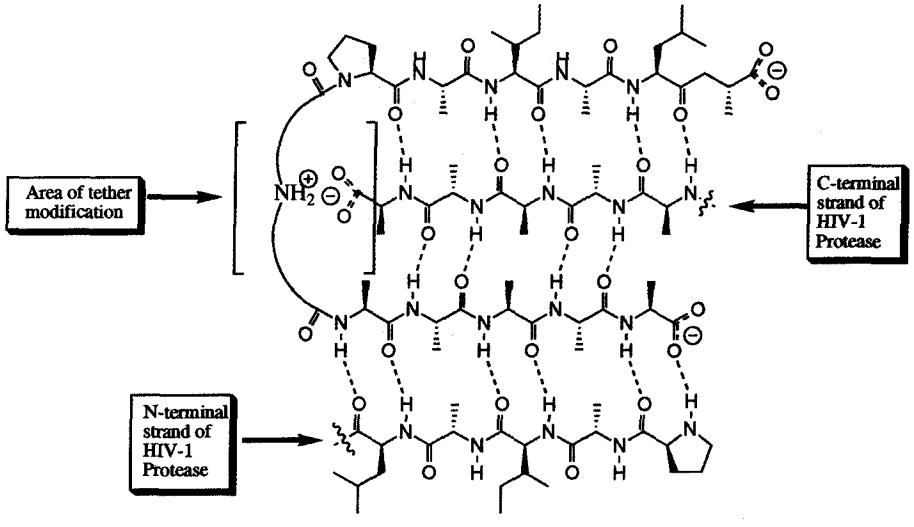
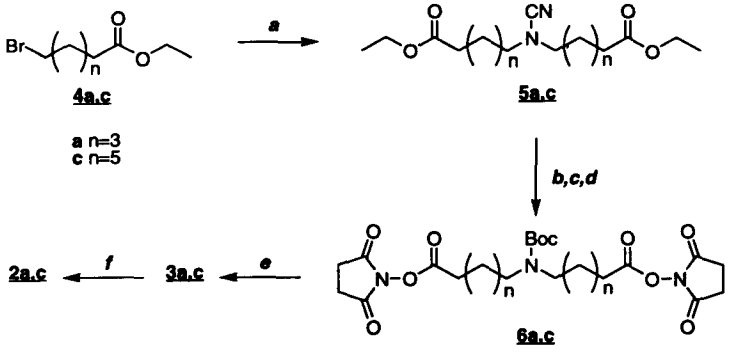


Figure 1. Design of tethers containing amino-functionality for enhanced interaction with carboxyl terminus (interior side chains omitted for clarity, backbone H-bonds indicated with dashed lines).

synthetic route that proceeds through the Boc-protected agents **3a–c** would also generate another set of control compounds to address the role of hydrophobicity in the inhibitors (Figure 1).

Synthesis of inhibitors

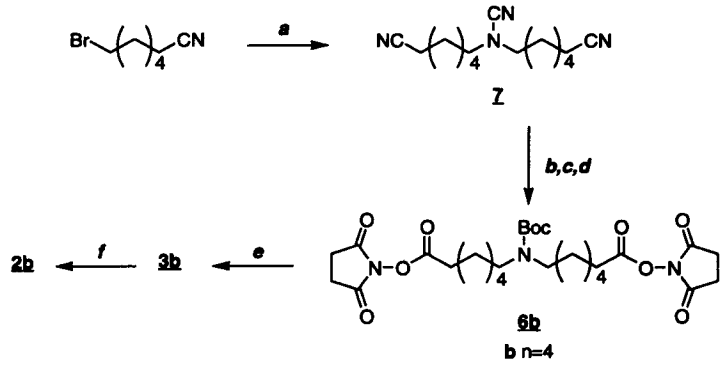
Conversion of **4** to the cyanamide **5** was easily achieved with sodium cyanamide in very good yields (91%) (Scheme 1). Hydrolysis was achieved by refluxing **5** with 3 M NaOH for 4 hours; shorter reflux times led to ester saponification without hydrolysis of the cyanamide. The resulting amine was reacted without further purification with di-*tert*-butyl dicarbonate, followed by removal of the dioxane *in vacuo* and neutralization with 1 M HCl. The crude diacids were treated with N-hydroxysuccinimide, EDCI, and DMAP in methylene chloride to afford **6** in moderate yields (48–75%).



Scheme 1. Synthesis of amine tethers **2a,c**: (a) Na_2NCN, DMF; (b) NaOH, MeOH, H_2O; (c) $(Boc)_2O$, dioxane, NaOH, H_2O; (d) EDCI, NHS, cat. DMAP, CH_2Cl_2; (e) STLNF, PQITLW, 2 eq. DIEA, DMSO 60°C 3 days; (f) TFA, CH_2Cl_2.

Starting with the 7-bromoheptanenitrile, the conversion to the cyanamide was achieved through an identical pathway to the formation of **6a,c** although somewhat lengthier hydrolysis times (reflux of 6 hours) were required to form **6b** (Scheme 2). Coupling of **6a–c** to the peptides STLNF and PQITLW produced a mixture of STLNF disubstituted on the tether, PQITLW disubstituted on the tether, and

the desired compound **3a–c** in an approximate 1:1:2 ratio. This mixture was purified by reverse phase HPLC to obtain **3a–c**. Deprotection with trifluoroacetic acid in methylene chloride (1:1 v/v) afforded **2a–c**.



Scheme 2. Synthesis of amine tether **2b**: (a) Na_2NCN, DMF; (b) NaOH, MeOH, H_2O; (c) $(Boc)_2O$, dioxane, NaOH, H_2O; (d) EDCI, NHS, cat. DMAP, CH_2Cl_2; (e) STLNF, PQITLW, 2 eq. DIEA, DMSO 60°C 3 days; (f) TFA, CH_2Cl_2.

Evaluation of inhibitors

To test inhibition, we used the fluorogenic substrate developed by Toth and Marshall.[6] The substrate Abz-Ile-Nle-Phe(p-NO_2)-Gln-Arg-NH_2, self-quenches with little background fluorescence when excited at 337 nm. When cleaved, the fluorescence at 410 nm increases 6-fold, and is an indication of reaction progress. PR was incubated with the inhibitor dissolved in DMSO, and the mixture was added to the substrate solution to start the reaction. The increase in fluorescence over time was compared to controls of preincubated PR with DMSO. IC_{50} values were obtained for the Boc protected amines **3a–c** and the free amines **2a–c** (Table 1).

Installation of a hydrophilic amine **2a–c**, which is cationic under assay conditions, decreased the effectiveness of the inhibitors as compared to the simple methylene tethers. It was noted that amines all had a roughly 10-fold decrease in effectiveness, regardless of the chain length. The amines protected as the hydrophobic Boc derivatives **3a–c** regained most, but not all, of their inhibitory capabilities. In the case of the protected amines, there was an approximate 2-fold decrease in effectiveness, presumably due to the bulky Boc protecting group.

Although originally unexpected, this suggests that hydrophobic interactions may drive inhibitor–protease association, with hydrogen bonding or electrostatic interactions playing a much smaller role. The length variation follows our original studies of methylene tethers showing optimal chain length of 14 atoms separating the peptides.[5] This study shows that 13 atom spacing with the amines and their derivatives are the most effective, but the 11 atom spacer is much less effective than the 15 atom spacer indicating that crosslinked peptides require the same tether length, regardless of its identity.

This study shows that the hydrophobicity of the tether is a crucial requirement in the dissociative inhibition of HIV-1 PR. In the original design of our tethers, computer modelling suggested an optimal chain length of 11 atoms, but subsequent results indicated that the length requirement was

Table 1. IC_{50} values (μM) of inhibitors of various tether lengths

Compound	Connecting Atoms in Tether						
	10	11	12	13	14	15	16
1	20	14	13	2.5	2	3.2	4
2	-	>130	-	27	-	40	-
3	-	20	-	6	-	5	-

underestimated. This finding along with the data presented here indicate that the tether may be wrapping around next to the hydrophobic Phe (99) side-chain. Upon re-examination of the PR crystal structure, it was noted that the amino acids Ile (3) and Leu (97) from each monomer form a hydrophobic wall which might make stabilizing interactions with the tether of our inhibitors.

Conclusions

Incorporation of hydrophilic, charged amino groups into the tethering moieties lowered the effectiveness of the interfacial PR inhibitors presumably because the tether preferred the hydrophobic pocket formed by the side chains Ile (3) and Leu (97) over its exposure to the solvent. Placement of a Boc-protecting group on the amine, however, led to inhibition being regained, presumably due to the necessity for hydrophobicity in the tether and hydrophobic binding to the PR monomer.

As has been shown in mutational studies of proteins, changing buried salt bridges to hydrophobic groups increases the rate of folding.[7] These data suggest that forming buried hydrogen bonds is difficult, and may be rate limiting in the acquisition of a protein's native state. Our system may behave similarly to proteins undergoing folding; burying the hydrophobic portions of the molecule may be the major driving force behind the formation of a PR monomer inhibitor complex.

Experimental

Diethyl ester cyanamide ***5a***

Ethyl 6-bromohexanoate (747 μl, 4.2 mmol) was added to a suspension of powdered Na_2NCN (164 mg, 1.9 mmol) in DMF (3 ml) and stirred at room temperature for 24 hours. The DMF was removed *in vacuo* and the resulting yellow oil was purified via flash chromatography (EtOAc:hexanes=50:50) to a pale yellow oil. Yield: 563 mg (81%). ^{1}H NMR ($CDCl_3$) δ (ppm)=1.14 (t, 6H, *J*=7.16 Hz), 1.31 (m, 6H, *J*=6.6 Hz), 1.55 (m, 8H, *J*=8.06 Hz), 2.20 (t, 4H, *J*=7.33 Hz), 2.87 (t, 4H, *J*=7.04 Hz), 4.01 (q, 4H, *J*=7.12 Hz). ^{13}C NMR ($CDCl_3$) δ (ppm)=14.66, 24.86, 26.37, 27.78, 34.42, 51.71, 60.68, 118.02, 173.78. CIMS [M+H]=327.

Diethyl ester cyanamide ***5c***

Ethyl 8-octanoate (938 mg, 3.7 mmol) was added to a suspension of powdered Na_2NCN (164 mg, 1.9 mmol) and NaI (28 mg, 0.19 mmol) in DMF (4 ml) and stirred at room temperature for 24 hours. The DMF was removed *in vacuo* and the resulting yellow oil was purified via flash chromatography (EtOAc:hexanes=15:85) to a pale yellow oil. Yield: 641 mg (90%). ^{1}H NMR ($CDCl_3$) δ (ppm)=1.18 (t, 6H, *J*=7.08 Hz), 1.27 (b, 12H), 1.55 (b, 8H), 2.22 (t, 4H, *J*=7.57 Hz), 2.90 (t, 4H, *J*=7.17 Hz), 4.05 (q, 4H, *J*=7.16 Hz). ^{13}C NMR ($CDCl_3$) δ (ppm)=14.72, 25.27, 26.76, 28.05, 29.31 34.70, 51.93, 60.65, 118.04, 174.20. CIMS [M+H]=383.

DiNHS ester ***6a***

A solution of **5a** (430 mg, 1.2 mmol) in NaOH (3 ml, 6 M) and EtOH (8 ml) was refluxed for 8 hours. The reflux condenser was then removed and the flask was heated until ammonia evolution had ceased (judged by moistened pH paper held over opening of flask), after approximately 10 minutes. The solution was then concentrated *in vacuo* to a volume of about 3 ml and washed with CH_2Cl_2 (2×5 ml). Water (2 ml) and dioxane (6 ml) were then added, and to the resulting solution di-*tert*-butylpyrocarbonate (77 6 mg, 3.6 mmol) was added and stirred at room temperature for 18 hours. The dioxane was then removed under reduced pressure, and the pH was adjusted to 7.3 with 0.1 M $KHSO_4$ and lyophilized to a white powder. A DMF:EtOAc solution (50:50, 10 ml) was used to suspend the powder as N-hydroxysuccinimide (342 mg, 3.0 mmol), EDCI (570 mg, 3.0 mmol), and DMAP (36 mg, 0.3 mmol) were added. After stirring for 48 hours at room temperature, the solution was concentrated *in vacuo*, and purified by column chromatography with EtOAc:hexanes (50:50) to yield an off-white solid. Yield: 479 mg (75%) ^{1}H NMR ($CDCl_3$) δ (ppm)=1.26 (s, 9H), 1.38 (m, 4H, *J*=6.88 Hz), 1.58 (m, 4H, *J*=7.32 Hz), 2.43 (t, 4H, *J*=7.33 Hz), 2.64 (s, 8H), 2.99 (bt, 4H, *J*=6.88 Hz).

^{13}C NMR ($CDCl_3$) δ (ppm)=14.58, 24.68, 25.97, 26.33, 28.81, 31.16, 47.12, 60.66, 79.43, 155.84, 168.94, 169.88. CIMS [M+H]=540.

DiNHS ester ***6b***

Following the procedure for the formation of **6a**, **5b** (219 mg, 0.8 mmol) was transformed into **6b** using appropriately scaled reagents. Yield: 200 mg (46%). ^1H NMR ($CDCl_3$) δ (ppm)=1.23 (m, 6H), 1.35 (s, 9H), 1.41 (m, 4H), 1.65 (m, 4H), 2.51 (t, 4H, *J*=7.3 Hz), 2.73 (s, 4H), 3.05 (b, 4H). ^{13}C NMR ($CDCl_3$) δ (ppm)=24.96, 26.04, 26.78, 28.93, 31.28, 47.33, 79.42, 156.00, 169.08, 169.80. CIMS [M+H]=568.

Dinitrile cyanamide 7

7-Bromoheptanitrile (730 µl, 4.9 mmol) was added to a suspension of powdered Na_2NCN (220 mg, 2.3 mmol) in DMF (2 ml) and stirred at room temperature for 24 hours. The DMF was removed *in vacuo* and the resulting yellow oil was purified via flash chromatography (EtOAc:hexanes=80:20) to a pale yellow oil. Yield: 497 mg (82%). ^1H NMR ($CDCl_3$) δ (ppm)=1.42 (m, 8H), 1.68 (m, 8H), 2.37 (t, 4H, *J*=7.1 Hz), 2.99 (t, 4H, *J*=7.2 Hz). ^{13}C NMR ($CDCl_3$) δ (ppm)=17.82, 25.68, 26.02, 27.56, 28.45, 51.89, 118.03, 120.24. CIMS [M+H]=261.

DiNHS ester ***6c***

Following the procedure for the formation of **6a**, **7** (332 mg, 0.9 mmol) was transformed into **6c** using appropriately scaled reagents. Yield: 43 mg (8%). ^1H NMR ($CDCl_3$) δ (ppm)=1.39 (b 12H), 1.44 (s, 9H), 1.74 (m, 4H), 2.60 (t, 4H), 2.83 (s, 4H), 3.10 (bt, 4H). ^{13}C NMR ($CDCl_3$) δ (ppm)=24.49, 25.58, 26.61, 28.50, 28.73, 28.86, 30.19, 46.95, 78.93, 155.59, 168.63, 169.20. CIMS [M+H]=596.

Boc-protected inhibitor ***3a***

To a solution of the peptides H_2N-Pro-Gln-Ile-Thr-Leu-Trp-COOH (20 mg, 26.4 µmole) and H_2N-Ser-Thr-Leu-Asn-Phe-COOH (15.3 mg, 26.4 µmole) in DMSO (5 ml) was added **3a** (15.7 mg, 29.1 µmole) and diisopropylethylamine (10 µl, 58.0 µmole). The solution was stirred at 60°C for 48 hours. The DMSO was passed through a 4 µm filter and purified by reverse phase HPLC, (CH_3CN:H_2O=25:75–60:40 with 0.1% TFA over 60 minutes). The compound was a single peak by analytical HPLC, (CH_3CN:H_2O=25:75–60:40 with 0.1% TFA over 30 minutes, R_t=22.8 min.) judged to be >95% pure. Yield: 6.9 mg (17%). ^1H NMR (d-DMSO) δ (ppm)=1.26 (s, 9H, Boc protecting group), 10.82 (s, 1H). FABMS, [M+H]=1547.4 (1647 calculated, observed loss of Boc group in the gas phase).

Boc-protected inhibitor ***3b***

Following the procedure for the formation of **3a**, **3b** was synthesized. The compound was a single peak by analytical HPLC, (CH_3CN:H_2O=25:75–60:40 with 0.1% TFA over 30 minutes, R_t=24.4 min.) judged to be >95% pure. Yield: 7.2 mg (16%). ^1H NMR (d-DMSO) δ (ppm)=1.27 (s, 9H, Boc protecting group), 10.92 (s, 1H). FABMS, [M+H]=1574.2 (1675 calculated, observed loss of Boc group in gas phase).

Boc-protected inhibitor ***3c***

Following the procedure for the formation of **3a**, **3c** was synthesized. The compound was a single peak by analytical HPLC, (CH_3CN:H_2O=25:75–60:40 with 0.1% TFA over 30 minutes, R_t=26.7 min.) judged to be >95% pure. Yield: 16.2 mg (17%). 1.26 (s, 9H, Boc protecting group), 10.77 (s, 1H). FABMS, [M+H]=1601.2 (1701 calculated, observed loss of Boc group in the gas phase).

Deprotected inhibitor ***2a***

To a 2 ml solution of TFA and CH_2Cl_2 (1:1), 5 mg of **3a** was added and stirred at room temperature for 30 minutes. After removal of the solvent by rotary evaporation the resulting residue was dissolved in 1 ml DMSO and purified by HPLC. The compound was a single peak by analytical HPLC,

(CH_3CN:H_2O=25:75–60:40 with 0.1% TFA over 30 minutes, R_t=15.2 min.) judged to be >95% pure with no sign of the Boc protecting group by NMR. Yield: 3.7 mg (79%). FABMS [M^+]=1548.3.

Deprotected inhibitor ***2b***

Following the procedure for the formation of **2a**, **2b** was synthesized. The compound was a single peak by analytical HPLC, (CH_3CN:H_2O=25:75–60:40 with 0.1% TFA over 30 minutes, R_t=16.3 min.) judged to be >95% pure with no sign of the Boc protecting group by NMR. Yield: 3.2 mg (68%). PDMS [M^+] 1573.2.

Deprotected inhibitor ***2c***

Following the procedure for the formation of **2a**, **2c** was synthesized. The compound was a single peak by analytical HPLC, (CH_3CN:H_2O=25:75–60:40 with 0.1% TFA over 30 minutes, R_t=17.4 min.) judged to be >95% pure with no sign of the Boc protecting group by NMR. Yield: 4.1 mg (87%). PDMS [M^+] 1604.0.

Concentration determination

Inhibitor concentrations were determined by UV absorbance at 280 nm (ε=5500 M^{-1} cm^{-1} for Trp in 6 M guanidine hydrochloride). 10 µl of a DMSO solution of the inhibitors was added to 990 µl of freshly filtered 6 M guanidine hydrochloride. Solutions were blanked with a 1% solution of DMSO in 6 M guanidine hydrochloride.

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