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Facile incorporation of urea pseudopeptides into protease substrate analogue inhibitors

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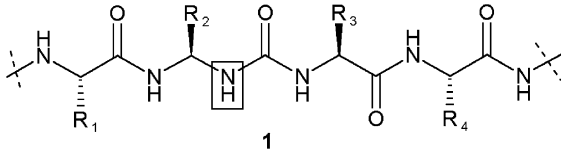
Dedicated to the memory of the late Professor Murray Goodman

Abstract—A new procedure that employs a one-pot, oxidative Hofmann rearrangement to incorporate a urea linkage into peptide backbones is detailed herein. This methodology was used to replace the scissile peptide bonds of [Leu^5]enkephalin and a hexapeptide HIV-1 protease substrate. The [Leu^5]enkephalin analogue was found to inhibit cleavage of hippurylhistidylleucine (HHL) by porcine kidney angiotensin-converting enzyme (PK-ACE) with a 0.88 mM IC_{50} value, comparable to the Michaelis constant of [Leu^5]enkephalin with the same enzyme. The HIV-1 protease substrate analogue was shown to inhibit HIV-1 protease with an $IC_{50} = 34\,\mu M$.

1. Introduction

With the arrival of the era of proteomics, a major concern has become the elucidation and the exploitation of protein function for the development of new diagnostics and therapeutics.[1] One major class of proteins is the protease family, enzymes responsible for signaling, tissue remodeling, and viral maturation, among other functions.[2] In many cases, although the substrate sequence for a protease may be known, only nonselective inhibitors may be available for its study. Herein we disclose a method for the rapid production of protease inhibitors from only the protease's substrate sequence. The development of selective protease inhibitors can assist the investigation of protein function and the development of lead compounds for antiviral drug development. In the case of HIV-1, protease inhibition has been shown to halt viral replication.[3]

Two main classes of protease inhibitors are transition-state analogues and nonhydrolyzable substrate analogues. In both cases, the production of protease inhibitors by the replacement of the scissile amide bond with linkages resistant to hydrolysis is one method used to harness the diversity of peptides for protease inhibition. Different strategies have emerged for this purpose, including substrate analogues (thioamides,[4] thiopyrrolidinones,[5] α-ketocarbonyls,[6] and (*E*)-olefins[7]) and transition-state analogues (*N*-hydroxyamides,[8] amino amides,[9] difluoromethyleneketone retroamides,[10] and *cis*-epoxides,[11]) as amide bond replacements in peptides. Aside from thioamide pseudopeptides, synthesis of these backbone modifications typically requires several additional synthetic manipulations, making synthesis of these species more tedious than that of peptides themselves and thereby limiting the utility of these backbone modifications.



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The approach described herein uses an oxidative Hofmann rearrangement of C-terminal peptide amides for the facile synthesis of a peptide bond replacement containing a urea linkage. Such peptidomimetics (e.g., **1**), which we have termed ureidopeptides, can be envisaged as a result of the insertion of a nitrogen atom between the α-carbon and the carbonyl of the scissile amide bond. Ureidopeptides retain a strong structural resemblance to their cognate peptides, as they differ by the

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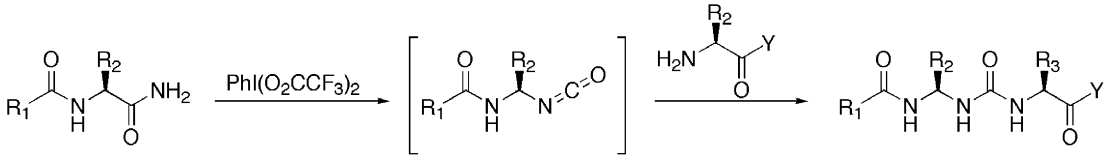


Figure 1. Oxidative Hofmann rearrangement of *N*-acylamino amide to form a ureidopeptide.

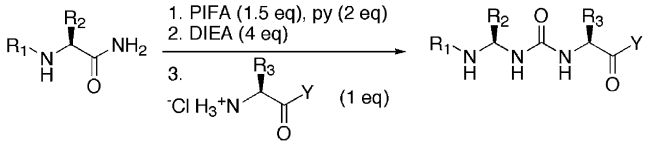
addition of a single backbone atom, but are nonetheless in principle inert to enzymatic hydrolysis. Use of this class of pseudopeptide provides a convenient strategy for the creation of active site protease inhibitors. Ureidopeptide monomers can be viewed as lower homologues of those in the oligoureas synthesized by Burgess et al.[12] and Schultz and co-workers,[13] but can be easily synthesized from commercially available monomers in a fashion analogous to standard peptide synthesis. Several previous examples of ureidopeptides have appeared in the literature, sometimes as the unexpected side product of acyl azide-based peptide couplings.[14]

This method uses a general one-pot synthesis of ureidopeptides in high yield and purity from commercially available protected amino amides and amino acids. This methodology has been tested by the synthesis and evaluation of two novel ureidopeptides, a [Leu^5]enkephalin analogue (**2**) and a novel ureidopeptide HIV-1 protease active site inhibitor (**3**). The ureidopeptide **2** was tested as an inhibitor of porcine kidney angiotensin-converting enzyme (PK-ACE), a surrogate for endogenous enkephalinases.

Babu and co-workers[15] and Guichard et al.[16] have recently synthesized urea peptidomimetics via a two-step process utilizing acyl azides in which the intermediate isocyanates were isolated prior to formation of the urea. In contrast, our one-pot synthesis of ureidopeptides involves an oxidative Hofmann rearrangement of protected amino amides using bis(trifluoroacetoxy)-iodobenzene (PIFA) under anhydrous conditions, followed by interception of the presumed isocyanate intermediate (Fig. 1) with an amine nucleophile. Loudon has previously shown that PIFA-induced Hofmann rearrangement of *N*-acylamino amides proceeds cleanly and with no epimerization.[17]. In addition, all but five amino acid side chains (Asn, Gln, Cys, Met, and Trp) were shown by Loudon to be compatible with PIFA.

A systematic survey of substrate, nucleophile, *N*-terminal protecting group and solvent was undertaken to investigate the scope of the PIFA-promoted Hofmann rearrangement in the synthesis of ureidopeptide analogues of dipeptides. Typically, a protected amino amide was treated with PIFA and pyridine under anhydrous conditions, the TFA salt neutralized using *N*,*N*-diisopropylethylamine (DIEA) and the amine nucleophile added. Typically, THF was used as the solvent for this reaction, although the reaction also proceeds well in a variety of other anhydrous solvents (CH_2Cl_2, CH_3CN, toluene, DMA). Optimized conditions used 1.5 equiv PIFA, 2.0 equiv pyridine, and 4.0 equiv DIEA, with THF being the optimal solvent. Products of high purity were isolated by flash chromatography and recrystallization. Using these conditions a number of ureidopeptide dipeptide analogues were synthesized in good yield (Table 1).

Table 1. Survey of substrates, nucleophiles, and *N*-terminal protecting groups used in the oxidative Hofmann rearrangement

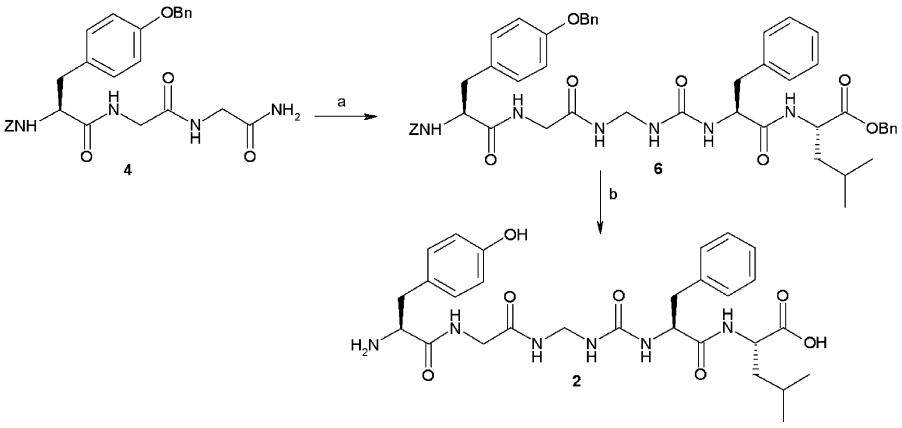


Entry	R_1	R_2	R_3	Y	Yield, %[a]
1	Cbz	H	$PhCH_2$	OMe	93
2	Cbz	CH_3	$PhCH_2$	OMe	83
3	Cbz	*i*-Bu	$PhCH_2$	OMe	85
4	Cbz	$PhCH_2$	*i*-Bu	OBn	79
5	Cbz	CH_3	$PhCH_2$	NH_2	69
6	Cbz	$PhCH_2$	*i*-Bu	NH_2	78
7	Fmoc–	H	$PhCH_2$	OMe	83
8	Ac	$PhCH_2$	*i*-Bu	OBn	78
9	*t*-BuO_2C	$PhCH_2$	*i*-Bu	OBn	85
10	$C_3H_6O_2C$	$PhCH_2$	*i*-Bu	OBn	89
11	Cbz-Ala	*i*-Bu	$(CH_2)_4$	Phe-NH_2	96

[a] Yield of purified product.

A convergent strategy was chosen to accomplish the synthesis of the ureidopeptides to avoid the complication of deprotecting an α-amino urea. Z-Tyr(OBn)-Gly-Gly-NH_2 (**4**) and Phe-Leu-OBn (**5**) were synthesized in high yield by standard peptide coupling conditions.[18] The synthesis of compound **2** was accomplished by reacting amino amide **4** with PIFA and pyridine in THF at room temperature for 1 h, followed by addition of DIEA and **5**. The reaction was complete after 16 h, as shown in Scheme 1. Ureidopeptide **6** was purified by flash column chromatography and recrystallization, giving an isolated yield of 31%. The low yield of this coupling was attributable to partial oxidation of the benzyl ether of the tyrosine residue, as previously demonstrated by Spyroudis and Varvoglis.[19] Removal of the protecting groups from **6** was accomplished via hydrogenation in 1:1 methanol–methylene chloride over 4 h, followed by RP-HPLC purification, affording **2** in 79% yield.

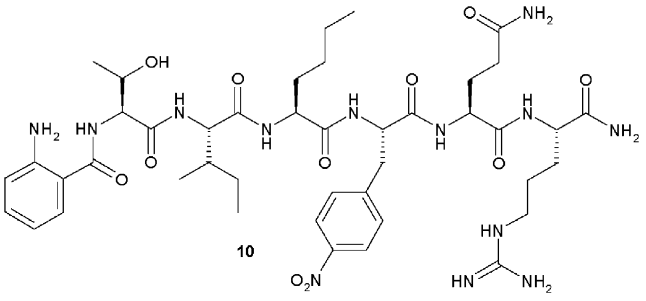
Ureidopeptide **2** was found to be stable to hydrolysis for greater than 24 h in aqueous buffer from pH 1 to pH 11, and stable to ACE-catalyzed proteolysis under conditions where [Leu^5]enkephalin was readily cleaved.[20] To demonstrate that the proteolytic stability of **2** was due to the lack of reactivity of the amide bond replacement and not simply because of loss of binding to ACE, a competition study between **2** and hippurylhistidylleucine

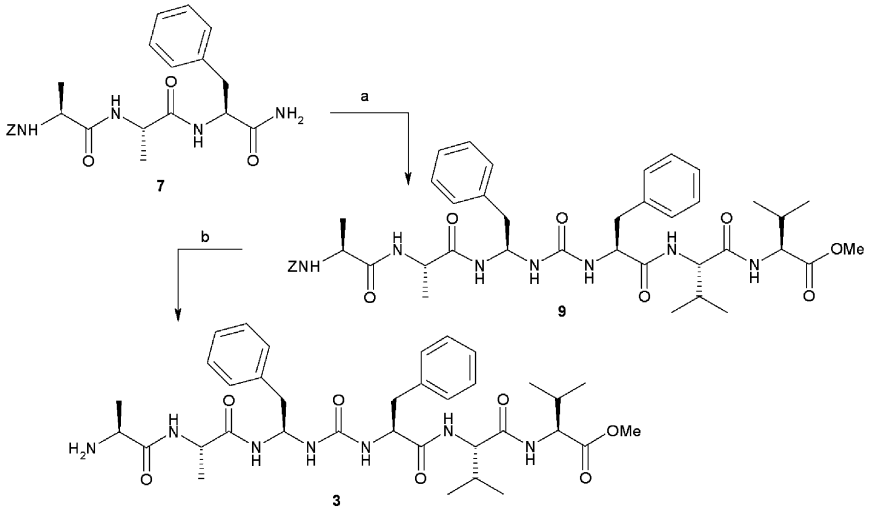


Scheme 1. Reagents and conditions: (a) (i) PIFA, pyridine, THF, 1 h; (ii) **5**, DIEA, 16 h, 31%; (b) H_2, $Pd(OH)_2/C$, 1:1 MeOH–DCM, 4 h, 79%.

(HHL), a known substrate for ACE, was conducted. The [Leu^5]enkephalin analogue **2** was found to inhibit ACE-catalyzed cleavage of HHL with an IC_{50} of 0.88 ± 0.06 mM, a value comparable to the Michaelis constant of [Leu^5]enkephalin (K_m = 1.0 mM).[21] These results indicate that the ureidopeptide **2** binds ACE with roughly the same affinity as the native peptide, suggesting that replacement of the Gly^3-Phe^4 amide bond with a urea linkage does not substantially affect recognition of the analogue **2** by ACE.

Having demonstrated that ureidopeptides are stable to hydrolysis and able to maintain active site recognition with [Leu^5]enkephalin analogue **2**, we next applied the same methodology to the design of an inhibitor for the HIV-1 protease. Lam et al. have previously described the use of cyclic ureas in HIV protease inhibition.[22] The sequence selected for our modification was AAFFVV-OMe, used previously by Dreyer et al. for the design of a hydroxyethylamine isosteric inhibitors.[23] Two tripeptides (**7** and FVV-OMe **8**) were synthesized using the standard peptide coupling conditions previously described. The synthesis of compound **3** was accomplished by reacting protected amino amide **7** with PIFA and *N*-methylimidazole in *N*,*N*-dimethylacetamide (DMA) at 0 °C for one and a half hours, followed by addition of DIEA and amine **8**, and allowing the reaction to warm to room temperature. *N*-Methylimidazole serves to neutralize the TFA produced from the reaction of PIFA with the primary amide; more basic amines were found by Loudon to promote the decomposition of PIFA.[13] DIEA is added in the second step to prevent protonation of the amine nucleophile by protonated *N*-methylimidazole. Under these conditions, coupled product **3** was obtained in 67% yield after 21 h





Scheme 2. Reagents and conditions: (a) (i) PIFA, *N*-methylimidazole, DMA, 0 °C, 1.5 h; (ii) DIEA, **8**, 0–25 °C, 21 h, 67%; (b) 5% Pd/C, NH_4HCO_2, MeOH, 60 °C, 5.5 h, 29%.

(Scheme 2). Removal of the benzyloxycarbonyl group of compound **9** was accomplished in 29% yield by catalytic transfer hydrogenation. The low yield of this step was attributed to poor solubility of the starting material **9** and loss of material during the HPLC purification of **3**.

Ureidopeptide **3** and the protected intermediate **9** were assayed for HIV-1 protease inhibition. A competitive binding assay[24] was used in which a solution of the inhibitor in dimethylsulfoxide (DMSO) at various concentrations was incubated with HIV-1 protease in a pH 5.52 buffer for 1 h at room temperature.[25] Inhibitor and literature substrate (**10**) were combined and fluorescence was measured. Assays showed moderate inhibition of HIV-1 protease by compound **3** (IC_{50} = 32.4 ± 0.5 μM). In contrast, the protected intermediate **9** showed negligible inhibition. It was found to have a maximum inhibition around 35%, highlighting that the free amine of inhibitor **3** is important in binding to HIV-1 protease.

2. Conclusion

The methodology reported here, employing the oxidative Hofmann rearrangement of C-terminal peptide amides, affords ureidopeptides using a two-step, one-pot transformation from commercially available starting materials. The reaction tolerates a variety of substrates, amine nucleophiles and solvents, and the urea linkage produced was determined to be stable over a wide pH range and to proteolysis. Replacement of the scissile amide bond of Leu-enkephalin, a substrate for ACE, with a urea resulted in the ureidopeptide ACE inhibitor **2**, which showed binding to ACE comparable to that of Leu-enkephalin itself. The ureidopeptide HIV-1 protease inhibitor **3** further illustrates the ability of ureidopeptides to inhibit proteases. This methodology provides a flexible strategy for rapidly synthesizing urea-containing peptidomimetic protease inhibitors, providing another tool for de novo design of inhibitors for proteolytic enzymes. Current efforts are underway to apply this methodology to other proteases of therapeutic interest.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2004.07.092.

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25. Compound **3** in DMSO (1 nM to 500 μM) was incubated for 1 h at 25 °C with HIV-1 protease (50 nM) in an aqueous buffer of 2 mM KH_2PO_4, 10% glycerol, 0.1% CHAPS, 1 mM EDTA, and 1 mM DTT, pH adjusted to 5.52 using Na_2HPO_4. Each concentration of inhibitor (60 μL) in protease was plated into 40 μL of 150 μM substrate **10**. Substrate cleavage was monitored at $\lambda = 415$ nm ($\lambda = 355$ nm excitation).