Rapid Diversity-Oriented Synthesis in Microtiter Plates for In Situ Screening of HIV Protease Inhibitors

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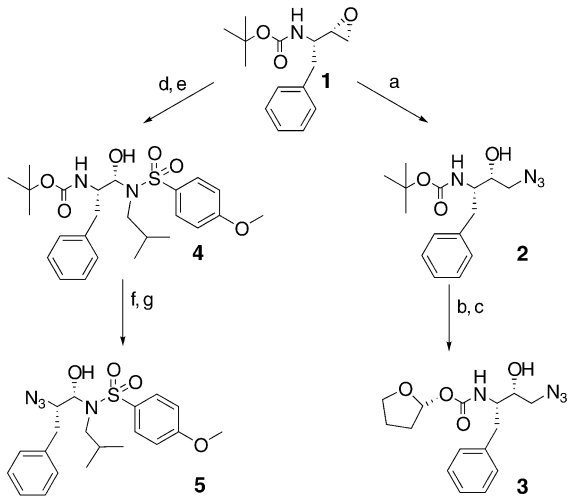
Since the early days of the discovery of HIV-1 protease (HIV-1 PR), this enzyme has been selected as an important target for the inhibition of viral replication.[1] The enormous effort over the past two decades to develop effective molecules that inhibit the HIV-1 PR has resulted in the discovery of drugs that have dramatically improved the quality of life and survival of the patients infected with HIV-1. To date there are six different HIV-1 PR inhibitors (PI) that are commercially available.[2] These drugs are administered in combination with the reverse transcriptase inhibitors in what is called "highly active anti-retroviral therapy (HAART)". Unfortunately, many drug-resistant and cross-resistant mutant HIV-1 PRs have been identified, thus hampering long term suppression of the virus and resulting in return of AIDS symptoms.[3] Therefore, the development of new protease inhibitors, which are efficacious against both the wild type and drug resistant HIV-1 PR and less prone to development of resistance, is urgently needed.

During the last decade, the number and throughput of biological assays of protease activity has notably increased. However, the high rates of HIV-1 PR mutation still outpace conventional drug discovery efforts, mostly because of limitations associated with identification of the lead structures and, to a greater extent, slow structure–activity profiling. While the former can be improved by rational design and computational studies, rapid synthesis of diverse analogues and their optimization still remains a challenge. We have recently developed a new strategy to facilitate the drug discovery process: diversity-oriented organic synthesis in microtiter plates followed by in situ screening without product isolation and protecting group manipulation. This strategy was demonstrated with the use of amide-forming reaction in a rapid identification of new potent HIV protease inhibitors.[4]

Click chemistry[5] has emerged as a strategy for the rapid and efficient assembly of molecules with diverse functionality on both laboratory and production scales. Enabled by a few nearly perfect reactions, it guarantees reliable synthesis of the desired products in high yield and purity. Modularity, selectivity, and wide scope make click chemistry ideal for achieving diversity in just a few steps and with no need for further purification.

Advantages of click chemistry in biological studies have recently been demonstrated in several applications: construction of fluorescent oligonucleotides for DNA sequencing,[6] in situ assembly of acetylcholinesterase inhibitors,[7] chemically orthogonal high fidelity bioconjugation,[8] and activity-based protein profiling in whole proteomes.[9] In principle, this type of chemistry is well suited for microscale synthesis and for biological screening in situ. To demonstrate its feasibility we have used the copper(I)-catalysed triazole formation for the synthesis of sugar arrays in the above mentioned microtiter plate format, followed by in situ screening of glycosyltransferase inhibitors and enzyme glycosylation.[10] Herein, we report an expedient approach to the discovery of novel HIV-1 PR inhibitors based on the latest advance in the copper(I)-catalyzed 1,2,3-triazole synthesis.[10, 11] This highly reliable process, which proceeds well in aqueous solvents and tolerates virtually all functional groups without the need for protection, made it possible to quickly generate the desired libraries of potential inhibitors and to screen them directly in microtiter plates, without any purification, against HIV-1 PR and its mutants.

The efficacy of hydroxyethylamine isosteres as transition-state mimics and as backbone replacements of amide bonds in the P1/P1′ position of aspartyl protease inhibitors has been well documented, most notably in incorporation in the structures of three commercially available drugs, amprenavir,[12] nelfinavir,[13] and saquinavir.[14] We, therefore, envisioned a library of compounds which retained this core, while diversifying the P2/P2′ residues to generate new inhibitors. Starting from the optically active epoxy amine **1**, two different azide cores were prepared as summarized in Scheme 1. Epoxy amine **1**[15] in H_2O/EtOH was



Scheme 1. *Preparation of the two azide cores* ***3*** *and* ***5****. a) NaN_3, EtOH/$H_2$0 60 °C, 2 h; b) 4 N HCl/dioxane; c) (S)-3-tetrahydrofuranyl* N*-oxysuccinimidyl carbonate, Et_3N; d) i-$BuNH_2$, MeOH; e)* p*-methoxybenzenesulfonyl chloride, K_2CO_3, CH_3CN, 3 h; f) 4 N HCl/dioxane; g) TfN_3, H_2O/CH_2Cl_2/MeOH, RT.*

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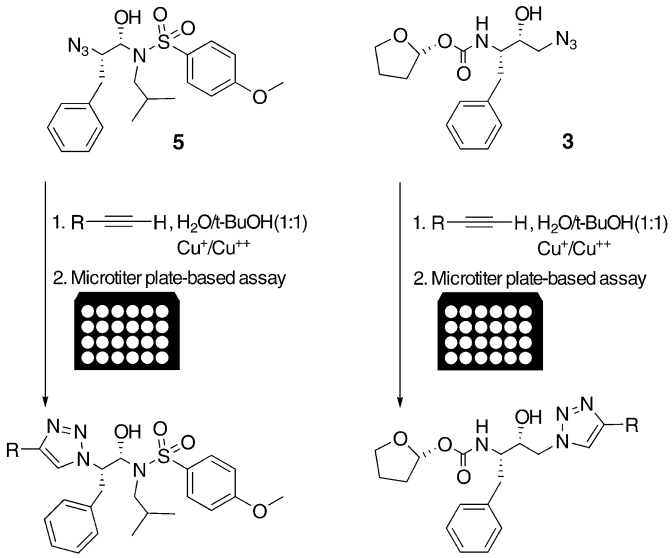
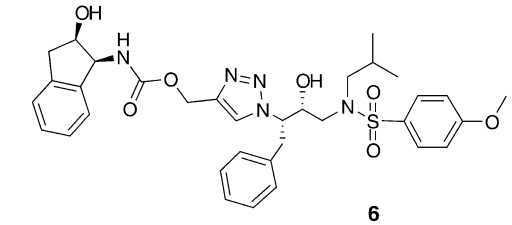
treated with NaN_3 at 60 °C for two hours to give **2** in 87% yield. Subsequent removal of the Boc group using 4 N HCl/dioxane followed by coupling of the free amine with (*S*)-3-tetrahydrofuranyl *N*-oxysuccinimidyl carbonate, furnished the desired product **3** in 83% overall yield. Starting from the same epoxy amine **1**, the second azide core **5** was prepared. Epoxy amine **1** was opened with isobutylamine followed by coupling of the secondary amine with *p*-methoxybenzenesulfonyl chloride to give **4**. Removal of the Boc group and application of the diazo transfer reaction,[16] gave the desired azide compound **5** in 76% overall yield.

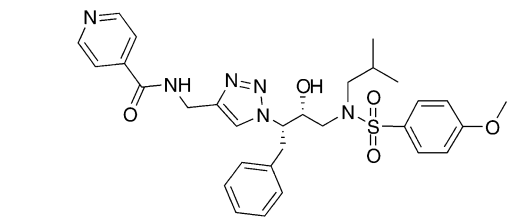
A solution of each core in *tert*-butyl alcohol was dispensed into fifty different vials, each containing a corresponding alkyne, catalytic amount of $CuSO_4$ and a piece of copper turning (Scheme 2). After incubation at room temperature for 48 h, LC-MS analysis indicated full consumption of the azide core and approximately quantitative formation of the cycloaddition product (Mw 400 – 720).

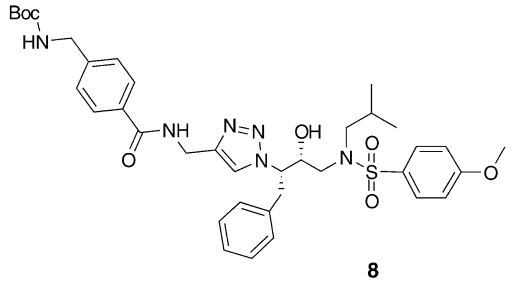


Scheme 2. *Principle of the triazole forming reaction to generate a library of HIV PR inhibitors, coupled with high throughput screening.*

Initially, all reactions were diluted to 100 nM into 200 μL wells of 96 microtiter plate and were assayed[17] for their inhibition activity against HIV-1 PR and three mutants (G48V, V82F, V82A). Wells that showed 50% inhibition of the enzyme activity were selected and diluted to 10 nM and screened again. Interestingly, at 100 nM none of the compounds derived from core **3** inhibited 50% of the activity of any of the four enzymes (HIV-1 PR, G48V, V82F, V82A). However, twelve compounds derived from the azide core **5** showed $>50\%$ inhibition activity against the four different enzymes. Furthermore, six of these compounds exhibited good activity against HIV-1 PR at 10 nM, most notably compounds **6**—**9**. The most active compounds, **6** and **8**, were synthesized, purified, and their IC_{50} and K_i values determined (Table 1). It should be mentioned that both cores, **3** and **5**, show a very high IC_{50} against the HIV-1 PR and its mutants (>3 μM, >1 μM, respectively).







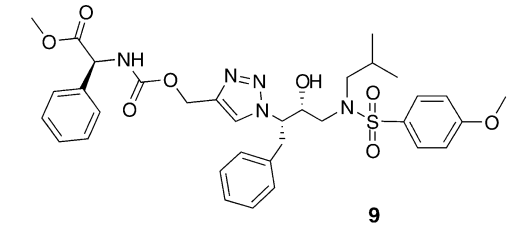


Table 1. *IC_{50} and K_i values for inhibitors **6** and **8**.*

	Inhibitor			
	6		**8**	
Enzyme	IC_{50} [nM]	K_i [nM]	IC_{50} [nM]	K_i [nM]
HIV PR	6 ± 0.5	1.7 ± 0.1	13 ± 0.5	4 ± 0.5
V82F	19 ± 1	10 ± 0.5	24 ± 1	13 ± 0.5
G48V	39 ± 1	22 ± 1	17 ± 1	9.7 ± 0.5
V82A	46 ± 2	27 ± 1	52 ± 2	30 ± 1

Many HIV-1 PR inhibitors have the disadvantage of low bioavailability because of their peptide character. Most inhibitors, including those that have been approved by the Food and Drug Administration (FDA), contain one or more amide bonds. The 1,4-triazole forming reaction allows for diversity through nonpeptidic linkage. The amide bond is well suited for hydrogen bonding with the binding site of the enzyme; however, it is not clear how the triazole moiety binds to the enzyme. In order to shed light on the binding mode of these linkages, compound **6** was computationally docked using AutoDock3.[18] Protein coordinates and coordinates for the water bound under the flaps were taken from PDB entry 1 hpv,[12] and coordinates for

compound **6** were built using ideal geometry. The docking simulation produced two conformations of approximately equal energy. One conformation placed the triazole in the position normally adopted by the peptide unit between P2 and P1 substituents in peptidomimetic compounds, with the central nitrogen of the triazole perfectly positioned to form a hydrogen bond with the water normally found under the protease flaps, as shown in Figure 1. This water also formed a hydrogen bond with the sulfonamide as seen in the crystallographic structure of amprenivir bound to HIV-1 protease.[12] The other conformation positioned the compound in a similar place, but with the triazole rotated by 180 degrees, allowing a slightly better fit of the triazole substituent but sacrificing the hydrogen bond with water. Further improvement of these inhibitors based on the> X-ray structures of HIV-1 PR bound to inhibitors **6** and **8** is currently under investigation.

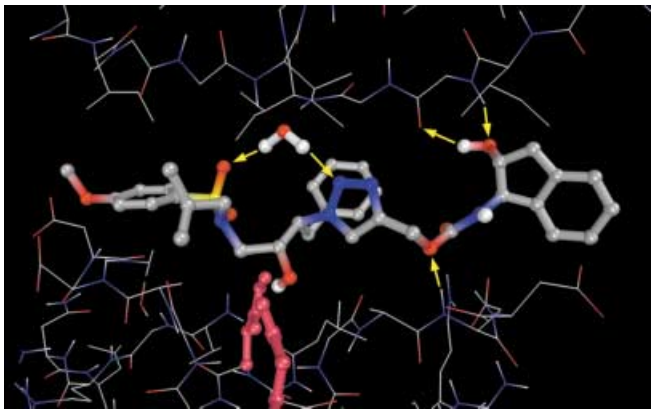


Figure 1. *Compound **6** computationally docked to HIV-1 protease. The best conformation is shown here, with the compound in balls-and-sticks at center, and the water shown above and the two catalytic aspartate residues in pink below. Key hydrogen bonds, shown with yellow arrows, are formed between the water and the compound and between main chain peptide atoms in the protein and the triazole substituent.*

In summary, Cu(I)-catalyzed stepwise variant of Huisgen's 1,2,3-triazole synthesis enabled rapid solution-phase preparation of a 100-member focused library, from which two novel inhibitors of HIV-1 PR have been identified by direct screening. This method should be widely applicable for efficient assembly of diverse compound collections that are useful in optimization of other P/P′ residue of protease inhibitors.

Experimental Section

General procedure for libraries assembly: A 10 mM solution of each core was prepared in *tert*-butyl alcohol, in which 200 μL of this solution were dispensed into fifty different vials, each containing a corresponding alkyne (1.2 equiv.), 200 μL of $CuSO_4$ (300 μM), and copper turning. The reactions were allowed to stand at room temperature for 48 hours. After this period of time, the reactions were analyzed by LC-MS and showed complete reaction based on the disappearance of the azide core and the appearance of a new peak corresponding to the mass of the cycloaddition product of the core and a specific alkyne (Mw 400 – 720).

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Keywords: click chemistry · enzyme · HIV protease · inhibitors · screening

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