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Epoxide opening in water and screening in situ for rapid discovery of enzyme inhibitors in microtiter plates

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Abstract—A method utilizing the strategy of epoxide opening by amine with water as co-solvent and screening in situ was developed for rapid discovery of protein inhibitors. Using this approach, HIV protease inhibitors with novel P1′ residues were identified in our study. This strategy should be applicable for the efficient assembly of diverse compound collections for inhibitors' discovery and optimization in other systems.

1. Introduction

We have recently developed a new strategy for rapid identification and optimization of protein inhibitors in microtiter plates.[1] It utilizes high yield organic reactions, which can be carried out in water or water miscible non-toxic solvents, amenable to microscale reactions and without protecting group manipulations. Therefore, the products can be assayed directly in situ without isolation and purification. Using this approach, a weak lead (>1 μM) can quickly be modified with a small set of building blocks to obtain a potent inhibitor. For example, by using the amide forming reaction, triazole forming reaction, TBAF-assisted N-alkylation, and ester bond formation, we have discovered potent inhibitors against HIV protease,[1] SARS 3CL protease,[2] α-fucosidase,[3] sulfotransferase,[4] and α-1,3-fucosyltransferase.[5]

Epoxides are versatile synthetic intermediates and a variety of reagents are known for the ring opening.[6] The resulting hydroxyethylamine products of epoxide aminolysis are important bioisosteres, which appeared in several FDA approved drugs.[7] Their efficacy as transition-state mimics and as backbone replacements of amide bonds in the P1/P1′ position of aspartyl protease inhibitors has been well studied.[8] Five out of seven currently approved HIV protease inhibitors, amprenavir,[9] nelfinavir,[10] saquinavir,[11] indinavir,[12] and atazanavir,[13] contain the hydroxyethylamine core structure. Opening of epoxides with amines has been reported in high efficiency by various methods with or without catalysts mostly in organic solvents. Only a few examples reported the use of water as a solvent for epoxide opening.[14] The nature and scope of this reaction make it very attractive for microtiter plate based reaction and in situ screening for the discovery of new inhibitors. To demonstrate the utility of this approach, we report here the development of epoxide opening in aqueous solution by primary amines in microtiter plates for the subsequent in situ identification of inhibitors with new P1′ residues against wild-type and mutant HIV proteases.

2. Results and discussion

We envisioned that the core **1**, which contains the tetrahydrofuran group and the epoxide moiety, can be used for our studies. The introduction of the substituted amine by epoxide opening would allow us to screen for the optimized P1′ residue. The synthesis of the epoxide core **1** started from introducing thiocresol to the

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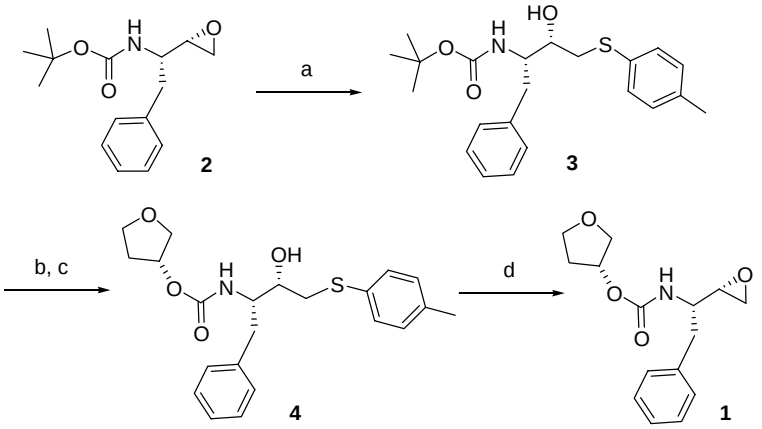
commercially available optically active epoxy amine **2** to mask the epoxide (Scheme 1). Subsequent removal of the Boc-protecting group using TFA–CH_2Cl_2 followed by coupling of the resulting free amine with (*S*)-3-tetrahydrofuranyl *N*-oxysuccinimidyl carbonate gave the epoxide precursor **4**. The desired epoxide core **1** was then re-generated by reacting with trimethyl oxonium tetrafluoroborate in anhydrous CH_2Cl_2, followed by aqueous K_2CO_3 treatment.[15]

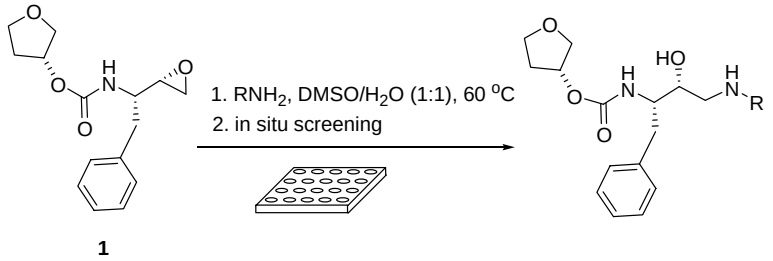
We then screened several epoxide ring opening reaction conditions with the synthesized epoxide core **1**. An alkyl amine, isobutyl amine, and a less nucleophilic aromatic amine, aniline, were used to react with the epoxide core under reaction conditions, either with only solvent: 2-propanol; DMF; H_2O–DMSO (4:1); or with added catalyst: $CsOH{\cdot}H_2O$/DMF; $LiClO_4/CH_3CN$;[16] thiourea/CH_3CN;[17] $P(Bu)_3$/DMF–H_2O (9:1);[18] $Mg(ClO_4)_2$/DMF.[19] In each case, the epoxide was reacted with 8 equiv of amine, and the reaction was monitored by TLC for disappearance of the epoxide and by LCMS to confirm the product formation. After 2 h at 60 °C, reactions were completed in 2-propanol, H_2O–DMSO (4:1) or $LiClO_4/CH_3CN$ to give desired aminolysis products for both alkyl and aromatic amines. Encouraged by the results, we decided to simply use H_2O–DMSO as our epoxide opening reaction condition, which had the advantage of precluding water-insoluble compounds in the in situ screening.

To test the generality of the method, a small library of epoxide core **1** was opened with 8 equiv of 20 structurally diverse primary amines in DMSO–H_2O (1:1) at 60 °C in 96-well microtiter plate. A complete consumption of the epoxide core **1** and formation of the hydroxyethylamine products were confirmed by LC–MS after 6 h. A more diverse library of 90 different primary amines was then synthesized under the same reaction condition and confirmed by LCMS (Scheme 2).

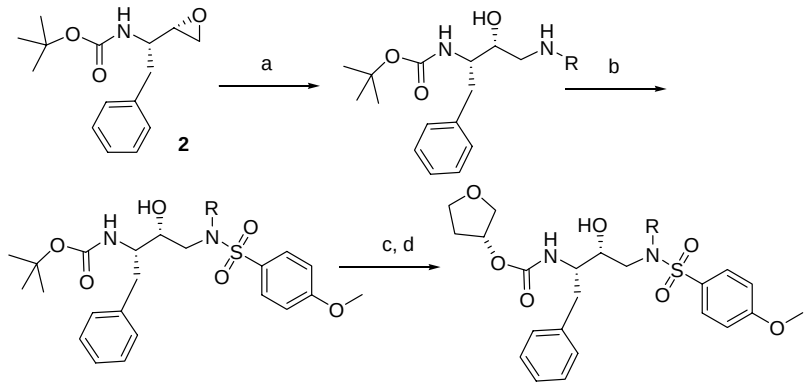
The reaction product in each well, which at this stage lacks the P2′ residue, was then diluted to 50 μM into another 96-well microtiter plate and assayed for their inhibition activity against HIV-1 PR and its variant with V82A mutation at S1/S1′ site.[20] Wells that showed over 50% inhibition of the enzyme activity for the wild-type or mutant proteases were selected and screened again at 10 μM. The whole process of synthesis, screening, and selection was done in less than 10 h. Two compounds with either 2-amino-*p*-cresol or 4-phenoxyaniline substituents showed good activity against both the wild-type and the V82A mutant proteases. Interestingly, the 4-phenoxyaniline substituent is very similar to the P1′ of the latest FDA approved HIV protease inhibitor—atazanavir.[13] The compound with the isobutyl amino substituent, which was presented in amprenavir,[9] showed poor inhibition from the screening. The two bulkier substituents were selected from the screening for the V82A mutant suggests that new structures with better fittings for the larger S1′ in the mutant protease can be identified. We synthesized pure inhibitors **5**–**7** with the same P2′ residue as that in amprenavir (Scheme 3),[21] and the K_i values of the synthesized inhibitors were determined (Table 1). Surprisingly, inhibitors



Scheme 1. Preparation of the epoxide core **1**. Reagents: (a) HSTol, KOH, EtOH (82%); (b) TFA–CH_2Cl_2 (1:1); (c) (*S*)-3-tetrahydrofuranyl *N*-oxysuccinimidyl carbonate, NEt_3, MeCN (85%, 2 steps); (d) Me_3OBF_4, CH_2Cl_2, then K_2CO_3, H_2O (62%).

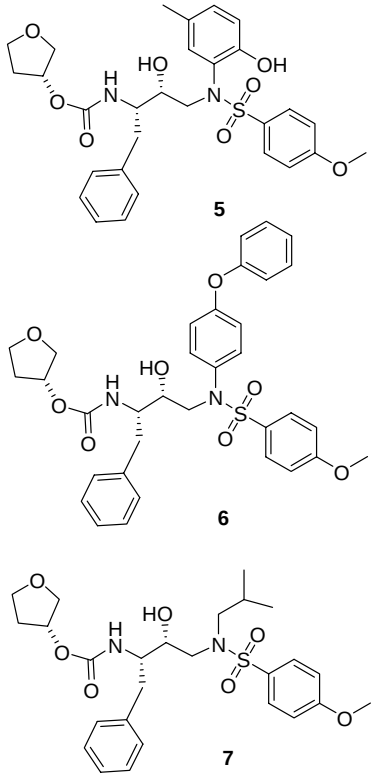


Scheme 2. Epoxide ring opening reaction with amines to form the hydroxyethyl amine isosteres for screening in situ of HIV protease inhibitors.



Scheme 3. A general procedure for the synthesis of pure HIV PR inhibitor. Reagents and conditions: (a) RNH_2, *i*-PrOH, 60 °C; (b) *p*-methoxybenzenesulfonyl chloride, pyridine, CH_3CN; (c) TFA–CH_2Cl_2 (1:1); (d) (*S*)-3-tetrahydrofuranyl *N*-oxysuccinimidyl carbonate, NEt_3, MeCN.

Table 1. K_i values for inhibitors **5**–**7**



	Inhibitor	HIV PR	V82A
K_i (nM)	**5**	3 ± 0.6	8 ± 1
	6	8 ± 1	13 ± 3
	7	3 ± 0.8	8 ± 2

5 and **6** have large P1′ residues, thus potentially could fit better to the large S1′ site in the V82A mutant, and showed comparable K_i values to compound **7**, which was similar to amprenavir and was used as our positive control. This indicates that optimizing the P2′ residue of the newly discovered leads with a novel P1′ residue may still be needed to obtain better inhibitors.

3. Conclusions

In summary, epoxide opening by amines enabled rapid preparation of a focused library of peptide isosteres in microtiter plates. Utilizing water as the solvent allowed the water-insoluble compounds to be excluded from the screening. From the in situ screening against wild-type and mutant proteases, two compounds with novel P1′ substituents were identified. This method should be widely applicable for the efficient assembly of a diverse structural collection for inhibitor discovery and optimization in other systems.

4. Experimental

4.1. Synthesis of epoxide core 1

Compound **1**: a solution of *p*-thiocresol (727 mg, 5.85 mmol) and potassium hydroxide (269 mg, 4.79 mmol) in ethanol (10 mL) was stirred at room temperature for 10 min and cooled to 0 °C. A solution of (2*S*,3*S*)-1,2-epoxy-3-(Boc-amino)-4-phenylbutane (1.4 g, 5.32 mmol) in ethanol (10 mL) was then added and stirred at room temperature for 3 h. The solvent was removed under reduced pressure. The crude product was treated with TFA–CH_2Cl_2 (1:1, 40 mL) and stirred at room temperature. After 1 h, toluene was added and the reaction was concentrated in vacuo. The crude product was then treated with freshly prepared (*S*)-3-tetrahydrofuranyl *N*-oxysuccinimidyl carbonate (2 equiv) and dry triethylamine (5 equiv) in dry CH_2Cl_2. The reaction was stirred at room temperature overnight under argon and then concentrated under reduced pressure. The product was purified by silica gel column chromatography (EtOAc–hexane = 1:4) to give a purified product (1.48 g, 70%). A solution of the resulting product (810 mg, 2.02 mmol) in dry CH_2Cl_2 (15 mL) was treated with Me_3OBF_4 (598 mg, 4.04 mmol) under argon with ice bath and stirred at room temperature for 2 h. A solution of K_2CO_3 (335 mg, 2.42 mmol) in water (4 mL) was added to the reaction mixture and stirred at room temperature for 1 day. After the reaction was finished, the mixture was extracted with CH_2Cl_2 and the organic

layer was washed with brine. The solution was dried (Na_2SO_4) and concentrated under reduced pressure. The product was purified by silica gel column chromatography (EtOAc–hexane = 1:3) to give compound **1** (347 mg, 62%). ^{1}H NMR (500 MHz, $CDCl_3$, 330 K): δ = 7.34–7.31 (m, 2H), 7.29–7.25 (m, 1H), 7.21 (d, J = 7.4 Hz, 2H), 5.19–5.17 (m, 1H), 4.65 (s, 1H), 3.87–3.71 (m, 5H), 2.97 (d, J = 5.2 Hz, 1H), 2.94–2.93 (m, 1H), 2.81 (t, J = 4.8 Hz, 1H), 2.76 (s, 1H), 2.12–2.08 (m, 1H), 1.98–1.94 (m, 1H), 1.63 (s, 1H); HRMS (ESI) m/z calcd for $C_{15}H_{20}NO_4$ $[M+H]^+$: 278.1387. Found: 278.1392.

4.2. General procedure for library synthesis and screening

An 18 mM solution of epoxide core was prepared in DMSO–H_2O (1:1). 100 μL of this solution was dispensed into each well of a 96-well microtiter plate, which also contained the corresponding amine (8 equiv). The reactions were allowed to stand in a 60 °C oven 6 h and analyzed by LC–MS. The screening of synthesized inhibitors was done following reported procedures.[20] AB2,[22] a reported potent inhibitor, was used as a positive control and DMSO was used as a negative control.

4.3. General procedure for inhibitor synthesis

A 100 mM solution of *tert*-butyl [*S*-(*R**,*R**)]-(−)-(1-oxiranyl-2-phenylethyl)carbamate (**2**) (1 equiv) in 2-propanol was treated with an amine (6 equiv) and stirred at 60 °C overnight. The reaction mixture was then concentrated in vacuo and purified by silica gel column chromatography. The resulting product was then dissolved in dry CH_3CN (100 mM) and were added dry pyridine (1.5 equiv) and *p*-methoxybenzenesulfonyl chloride (1 equiv) under argon. The reaction mixture was stirred at room temperature overnight, then concentrated in vacuo, and purified by silica gel column chromatography. The product was treated with TFA–CH_2Cl_2 (1:1) and stirred at room temperature. After 1 h, toluene was added and the reaction was concentrated in vacuo. The crude product was then treated with freshly prepared (*S*)-3-tetrahydrofuranyl *N*-oxysuccinimidyl carbonate (2 equiv) and dry triethylamine (5 equiv) in dry CH_2Cl_2. The reaction was stirred at room temperature overnight under argon, and then concentrated in vacuo and purified by silica gel column chromatography to give the pure inhibitor.

Compound **5**: ^{1}H NMR (500 MHz, $CDCl_3$, 330 K): δ 7.58 (d, J = 8.5 Hz, 2H), 7.45 (s, 1H), 7.28–7.24 (m, 2H), 7.22–7.19 (m, 1H), 7.16–7.15 (m, 2H), 7.00 (d, J = 8.1 Hz, 1H), 6.93 (d, J = 8.5 Hz, 2H), 6.88 (d, J = 8.4 Hz, 1H), 5.11–5.06 (m, 2H), 3.91–3.81 (m, 6H), 3.76–3.73 (m, 2H), 3.61 (d, J = 10.7 Hz, 1H), 2.96–2.92 (m, 1H), 2.82–2.78 (m, 1H), 2.11 (s, 3H), 2.11–2.04 (m, 1H), 1.95–1.92 (m, 2H); HRMS (ESI) m/z calcd for $C_{29}H_{35}N_2O_8S$ $[M+H]^+$: 571.2109. Found: 571.2107.

Compound **6**: ^{1}H NMR (500 MHz, $CDCl_3$, 330 K): δ 7.51 (d, J = 8.8 Hz, 2H), 7.36 (t, J = 8.1 Hz, 2H), 7.30–7.26 (m, 2H), 7.23–7.14 (m, 4H), 7.03 (d, J = 8.1 Hz, 2H), 6.97 (d, J = 8.8 Hz, 2H), 6.91 (dd, J = 8.8, 14.7 Hz, 4H), 5.09 (s, 1H), 5.02 (d, J = 8.8 Hz, 1H), 3.86 (s, 3H), 3.82–3.72 (m, 4H), 3.61–3.59 (m, 2H), 3.53 (d, J = 4.8 Hz, 1H), 3.00–2.97 (m, 1H), 2.92–2.87 (m, 1H), 2.9–2.04 (m, 1H), 1.91–1.89 (m, 1H); HRMS (ESI) m/z calcd for $C_{34}H_{37}N_2O_8S$ $[M+H]^+$: 633.2265. Found: 633.2262.

Compound **7**: ^{1}H NMR (500 MHz, $CDCl_3$, 330 K): δ 7.70 (d, J = 8.8 Hz, 2H), 7.30–7.27 (m, 2H), 7.23–7.19 (m, 3H), 6.97 (d, J = 8.8 Hz, 2H), 5.10–5.07 (m, 2H), 3.87–3.81 (m, 7H), 3.77–3.74 (m, 2H), 3.61–3.58 (m, 1H), 3.14–3.09 (m, 1H), 3.04–3.00 (m, 2H), 2.96–2.92 (m, 1H), 2.88–2.79 (m, 2H), 2.10–2.04 (m, 1H), 1.92–1.89 (m, 1H), 1.86–1.81 (m, 1H), 0.90 (d, J = 6.6 Hz, 3H), 0.86 (d, J = 7.0 Hz, 3H); HRMS (ESI) m/z calcd for $C_{26}H_{37}N_2O_7S$ $[M+H]^+$: 521.2316. Found: 521.2317.

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