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# Design, synthesis, and structure-activity relationship study of epoxysuccinyl-peptide derivatives as cathepsin B inhibitors

Xiaoye Zhang[a] · Xiaohong Yang[a] · Hongqiang Wang[a] · Song Li[b] · Kun Guo[c] · Dan Jiang[b] · Junhai Xiao[b,*] · Di Liang[a,*]

[a] *School of Pharmaceutical Sciences, Jilin University, Changchun 130021, China;*

[b] *Laboratory of Computer-Aided Drug Design & Discovery, Beijing Institute of Pharmacology and Toxicology, Beijing 100850, China;* [c] *Shaanxi University of Chinese Medicine, College of Humanities and Management, Century Avenue, The New District of Xi'an-Xianyang, Shaanxi Province 712046, China*

Corresponding author:

Di Liang, School of Pharmaceutical Sciences, Jilin University, Changchun 130021, China;

E-Mail: zxyxiaoye@foxmail.com; liangdi123@sina.com;

Junhai Xiao, Laboratory of Computer-Aided Drug Design & Discovery, Beijing Institute of Pharmacology and Toxicology, Beijing 100850, China;

E-Mail: xiaojunhai@139.com.

**Abstract**

Cathepsin B is a lysosomal cysteine protease involved in many diseases. The present research demonstrates that derivatives of epoxysuccinyl-peptide are effective and selective cathepsin B inhibitors. We synthesized a series of epoxysuccinyl-peptide derivatives based on the well-known cathepsin B inhibitor E64d. Specifically, we substituted the 2-methylpropane group at the $R^1$ position of E64d with a sulfane, such as ethyl(methyl) sulfane or benzyl(methyl) sulfane. We also designed and synthesized a library of molecules with various substituents at the $R^2$ position of E64d to replace 2-methylbutane. By studying the structure-activity relationships of these newly synthesized molecules as cathepsin B inhibitors, we demonstrated that substituting ethyl(methyl) sulfane for 2-methylbutane ($R^2$) of E64d improves the inhibitory activity and selectivity for cathepsin B inhibition. Our new cathepsin B inhibitors were highly effective and selective.

**Keywords**

Cathepsin B, lysosomal cysteine protease, structure-activity relationship, inhibitory activity, selectivity.

## Introduction

In modern society, cancer is a major threat to human health around the world. Although most patients who are diagnosed with cancer are recommended to undergo a surgical procedure to remove the tumors, a total resection is rarely achieved. It is necessary to follow the surgery with extensive radio- and/or chemotherapies to control the residual cancer cells. In addition, the total management of metastatic cancer cells is challenging; thus, a very high recurrence rate of cancers usually occurs [1)]. Cathepsin B is a member of the lysosomal cysteine protease family encoded by the cathepsin gene in humans. It is expressed in many organs, where it is involved in various aspects of immune responses, in the development and proliferation of various cell types, as well as in tumor invasion and metastasis [2)]. Emerging evidence suggests that elevated levels of cathepsin B are present in many tumor tissues [3, 4)]. Therefore, cathepsin B has been selected as a potential target for the treatment of a variety of human cancers, including kidney cancer, lung cancer, and liver cancer [5, 6)]. In order to achieve effective cancer treatment, it is of great importance to locate the active site of cathepsin B and to develop specific inhibitors. Typically, the active site of cathepsin B is divided into multiple subsites. Potent inhibitors of cathepsin B simultaneously bind to the $S_1'$ and $S_2'$ subsites [7)].

In the past few years, extensive work has been conducted to develop effective cathepsin B inhibitors for potential cancer treatment [4)]. For example, Katunuma has reported the synthesis of a derivative of the epoxysuccinyl-peptide cathepsin B inhibitor by covalently linking its ethylene oxide with the thiol group of the Cys29 residue of cathepsin B [8)]. In addition, Bogyo *et al*. have synthesized two interesting compounds: E64d and CA-074 (**1** and **2** as shown in Figure **1**) [9)]. Both are derivatives of the epoxysuccinyl-peptide and readily bind to the $S_1'$-$S_2'$ subsites of cathepsin B as highly selective inhibitors [10, 11)]. The selectivity of E64d and its related analogs has been determined to be due to efficient hydrogen-bond interactions

between a free carboxylic acid on the inhibitor and two histidine residues in the occluding loop structure found in cathepsin B [12].

Here, we describe the design and synthesis of highly selective and effective cathepsin B inhibitors based on epoxysuccinyl-peptide derivatives by applying different substituents at the $R^1$ and $R^2$ positions of E64d (Figure **2**). When we design a new chemical structure to replace the Leucine (Leu) of E64d, the principle is to maintain the inhibitory activity of the new molecule to Cathepsin B while reducing its activities to other Cathepsins (such as Cathepsin K, Cathepsin L) by different substitutions at $R^1$ and $R^2$ positions. Previously Watanabe *et al.* reported the synthesis of a $R^1$ derivative with oxygen atom substitutes as an inhibitor of epoxysuccinyl-peptide cathepsin B inhibitor [13], Sadaghiani and coworkers also synthesized a similar epoxysuccinyl-peptide cathepsin B inhibitor with a phenyl group at the $R^1$ position.[9] Both molecules however failed to maintain the overall inhibition and selectivity to Cathepsin B. Here we successfully synthesized a series of E64d derivatives with Methionine (Met, contains sulfur) substitute at $R^1$ position. As compared to Leu, Met substitute possesses very similar characteristics in steric hindrance, size, and chain length. It is thus hypothesized the substitution with Met will result in minimal effect on its inhibition of Cathepsin B. As for Cysteine, it is a bulky group as compared to Leu and Met and is expected to create a larger steric hindrance. We hope through this substitution in $R^1$ of E64d to understand how hindrance increase impact its overall selectivity and inhibitory activities. In addition, we also carefully selected 5 substituents at $R^2$ with different structure and size. We hope through this series of comparison, we was able to understand their selectivity and structure-activity relationships as Cathepsin B inhibitors. Interestingly, we found that the ethyl(methyl) sulfane or benzyl(methyl) sulfane moiety at the $R^1$ position, replacing 2-methylpropane of E64d, had improved specificity and selectivity as a cathepsin B inhibitor.

## Materials and Experiments

General

Silica gel $60F_{254}$ 25 aluminum sheets (20 × 20 cm) from Merck KGaA were used for TLC. Silica gel (200–300 mesh, YanTai Huagong) was used for column chromatography. The $^1$H-NMR spectra (400 MHz) were recorded on a JNM-ECA-400 in DMSO and $CHCl_3$. Chemical shift values were reported in parts per million (δ) downfield from TMS as the internal reference. Unless otherwise specified: br, broad; s, singlet; d, doublet; t, triplet; q, quarter; m, multiplet; dd, double doublet. Mass spectra were obtained on an Agilent 1260-G6230A (USA).

Synthesis

Diethyl (2*S*,3*S*)- oxirane-2,3-dicarboxylate (**5**)

A 2-L, dry, round-bottom flask was charged with L-arginine (0.56 mol; 97.01 g) in 250 mL of warm water, to which a total of 1,078 mL of methanol solution containing 74.03 g of **3** (0.56 mol, (+/-)-*trans*-epoxysuccinic acid, from Alfa Aesar Chemical Co.) was then slowly added over 20 min; the mixture was stirred at room temperature overnight. A white precipitate was filtered and washed with 410 mL of MeOH-water (4:1) to furnish 83.50 g of crude **4**. Recrystallization of the crude material from 1,200 mL of MeOH-water (2:1) yielded 70.60 g of **4** as colorless prisms. The purified **4** (0.23 mol; 70.60 g) was then added to 67.90 g of 95% $H_2SO_4$ (0.69 mol) in 692 mL of EtOH, and the mixture was stirred for 4.5 h at room temperature. After removing the solvent under vacuum, the mixture was poured into 400 mL of deionized water and extracted with 400 mL of AcOEt by a separatory funnel. The combined organic layers were washed with 200 mL of $NaHCO_3$-brine and dried over $MgSO_4$. The residue was purified by silica column chromatography (1:4 AcOEt:petroleum ether), yielding 34.70 g of **5** (0.18 mol; 80%) as a colorless oil. $[\alpha]_D$ +110.5 (c = 1.12, EtOH).

$^1$H-NMR ($CDCl_3$, 400 MHz) δ: 1.31 (6H, t, J= 7Hz), 3.66 (2H, s), 4.28 (4H, dq, J= 7, 2Hz). ESI-MS: *m/z* = 189 $[M+H]^+$.

(2*S*,3*S*)-3-(ethoxycarbonyl)oxirane-2-carboxylic acid (**6**)

**5** (0.1 mol; 18.8 g) and 85% KOH (0.1 mol; 6.72 g) were mixed in a 150-mL flask. After adding 67 mL of EtOH at 4–6 °C, the mixture continued to stir at room temperature for 4 h. The mixture was concentrated under vacuum and then diluted with water and AcOEt. The combined organic phase was washed with 25 mL of 6 N HCl and dried over $MgSO_4$. Distillation of AcOEt yielded 12.01 g of **6** (0.075 mol; 75%) as a colorless oil. $^1$H-NMR ($CDCl_3$, 400 MHz) δ: 1.17–1.27 (3H, t, J= 7Hz), 3.72–3.74 (2H, s), 4.23–4.33 (2H, dq, J= 7, 2Hz), 10.56 (1H, s). ESI-MS: *m/z* = 161 $[M+H]^+$.

Ethyl (2*S*,3*S*)-3-(((*S*)-1-(hexylamino)-4-(methylthio)-1-oxobutan-2-yl)carbamoyl)oxirane-2-carboxylate (**11a**)

In a round-bottom flask containing **7** (Boc-L-methionine; 0.031 mol; 7.7 g), **8a** (hexylamine from Alfa Aesar; 0.031 mol; 3.13 g), and 1-hydroxybenzotriazole (0.031 mol; 4.19 g) in AcOEt, a solution of *N,N'*-dicyclohexylcarbodiimide (0.031 mol; 6.39 g) dissolved in AcOEt was added dropwise, and the mixture was stirred at 5 °C for 1.5 h. The solution was then warmed to room temperature and stirred for 2.5 h. The reaction mixture was filtered and washed with AcOEt. The organic layer was washed with 5% HCl and saturated $NaHCO_3$-brine solution, dried over $MgSO_4$, and filtered. Removal of the solvent yielded 9.99 g of **9a** (0.030 mol; 96%) as a colorless solid intermediate.

Next, to a solution of **9a** (0.012 mol; 3.99 g) in 15 mL of AcOEt, 10% HCl-AcOEt was added, and the mixture was stirred for 2.5 h at room temperature. The solution was concentrated under vacuum, and the residual volume was diluted with water and extracted

with AcOEt. The aqueous phase was alkalified with 25% NaOH (pH > 10) and washed with AcOEt. The combined AcOEt layers were washed with brine and dried over $MgSO_4$. Removal of AcOEt yielded 2.55 g of **10a** (0.011 mol; 90%) as a yellow oil, which was used directly in the next step.

Finally, in a round-bottom flask containing **6** (0.009 mol; 1.44 g), **10a** (0.009 mol; 2.09 g), and 1-hydroxybenzotriazole dissolved in 20 mL of AcOEt, *N,N'*-dicyclohexylcarbodiimide (0.009 mol; 1.85 g) dissolved in AcOEt was added dropwise. After stirring at 5 °C for 3 h, the solution was warmed to room temperature and stirred for another 15 h. The reaction mixture was then filtered and washed with AcOEt. The organic layers were combined, washed with 100 mL of 5% HCl (v/v) and 100 mL of $NaHCO_3$ saturated with brine, and then dried over $MgSO_4$. After filtration, removal of the solvent afforded the solid product **11a**. Recrystallization from EtOH yielded 1.49 g of pure **11a** (0.0037 mol; 42%) as a white powder. $[\alpha]_D$ +47.1 (c = 1.00, MeOH). $^1$H-NMR (DMSO, 400 MHz) δ: 0.82 (3H, t, J= 7Hz), 1.17–1.35 (11H, m), 1.79–1.88 (2H, m), 2.00 (3H, s), 2.36–2.40 (2H, m), 2.90–3.20 (2H, m), 3.61 (1H, d, J= 2Hz), 3.70 (1H, d, J= 2Hz), 4.16–4.17 (2H, m), 4.27–4.28 (1H, m), 8.00–8.02 (1H, t, J= 7Hz), 8.63 (1H, d, J= 8Hz). $^{13}$C-NMR (DMSO) δ: 170.1, 167.3, 164.8, 61.5, 53.0, 52.1, 51.2, 38.5, 31.9, 30.9, 29.5, 28.9, 25.9, 22.1, 14.6, 14.0, 13.9; ESI-MS: *m/z* = 375 $[M+H]^+$ 397 $[M+Na]^+$. HRMS calcd for $C_{17}H_{30}O_5N_2S$ $[M+Na]^+$: 397.1768, Found: 397.1768.

Ethyl (2*S*,3*S*)-3-(((*S*)-4-(methylthio)-1-oxo-1-((3-phenylpropyl)amino)butan-2-yl)carbamoyl)oxirane-2-carboxylate (**11b**)

Similarly, **11b** was synthesized by starting from **8b**, 3-phenyl-1-propylamine, as a white powder (52%). $[\alpha]_D$ +46.3 (c = 1.00, MeOH). $^1$H-NMR (DMSO, 400 MHz) δ: 1.17–1.27 (3H, m), 1.64–1.90 (4H, m), 2.00 (3H, s), 2.38-2.42 (2H, m), 2.53 (2H, m) 3.00–3.10

(2H, m), 3.61 (1H, d, J= 2Hz), 3.70 (1H, d, J= 2Hz), 4.16–4.17 (2H, m), 4.27–4.28 (1H, m), 7.17–7.27 (5H, m), 8.13 (1H, t, J= 7Hz), 8.63 (1H, d, J= 8Hz). $^{13}$C-NMR (DMSO) δ: 170.2, 167.2, 164.8, 141.6, 128.5, 128.5, 128.2, 128.2, 125.7, 61.4, 52.9, 52.1, 51.2, 38.1, 32.3, 31.9, 30.7, 29.9, 14.6, 13.8; ESI-MS: *m/z* = 409[M+H]$^+$ 431 [M+Na]$^+$. HRMS calcd for $C_{20}H_{28}O_5N_2S$ [M+Na]$^+$: 431.1610, Found: 431.1608.

Ethyl (2*S*,3*S*)-3-(((*S*)-1-((3-fluorophenethyl)amino)-4-(methylthio)-1-oxobutan-2-yl)carbamoyl)oxirane-2-carboxylate (**11c**)

**11c** was synthesized by starting from **8c**, 3-phenyl-1-propylamine, as a white powder (44%). [α]$_D$ +44.9 (c = 1.00, MeOH). $^1$H-NMR (DMSO, 400 MHz) δ: 1.20–1.27 (3H, t, J= 7Hz), 1.79–1.86 (2H, m), 2.00 (3H, s), 2.36-2.40 (2H, m), 2.70–2.75 (2H, m), 3.61–3.72 (4H, m), 4.16–4.17 (2H, m), 4.28–4.32 (1H, m), 7.02 (3H, m), 7.30 (1H, m), 8.17 (1H, t, J= 7Hz), 8.63 (1H, d, J= 8Hz). $^{13}$C-NMR (DMSO) δ: 170.2, 167.2, 164.8, 161.6, 142.8, 130.2, 125.2, 115.7, 113.2, 61.5, 53.2, 52.9, 52.1, 51.2, 34.7, 32.0, 29.9, 14.9, 13.9; ESI-MS: *m/z* = 413[M+H]$^+$ 435 [M+Na]$^+$. HRMS calcd for $C_{19}H_{25}O_5N_2SF$ [M+Na]$^+$: 435.1326, Found: 435.1324.

Ethyl (2*S*,3*S*)-3-(((*S*)-4-(methylthio)-1-oxo-1-(pentan-3-ylamino)butan-2-yl)carbamoyl)oxirane-2-carboxylate (**11d**)

**11d** was synthesized by starting from **8d**, 3-aminopentane, as a white powder (42%). [α]$_D$ +46.9 (c = 1.00, MeOH). $^1$H-NMR (DMSO, 400 MHz) δ: 0.80 (6H, t, J= 7Hz), 1.21–1.45 (7H, m), 1.79–1.90 (2H, m), 2.02 (3H, s), 2.36-2.42 (2H, m), 3.49–3.52 (1H, m), 3.61 (1H, m), 3.72 (1H, d, J= 2Hz), 4.16–4.17 (2H, m), 4.27–4.28 (1H, m), 7.77 (1H, t, J= 7Hz), 8.60 (1H, d, J= 8Hz). $^{13}$C-NMR (DMSO) δ: 170.5, 167.7, 165.2, 61.5, 52.8, 52.3,

52.1, 51.2, 31.9, 29.9, 27.4, 27.2, 14.6, 13.9, 10.9, 10.8; ESI-MS: *m/z* = 361[M+H]$^+$ 383 [M+Na]$^+$. HRMS calcd for $C_{16}H_{28}O_5N_2S$ [M+Na]$^+$: 383.1612, Found: 383.1612.

Ethyl (2*S*,3*S*)-3-(((*S*)-4-(methylthio)-1-oxo-1-((4-phenylbutyl)amino)butan-2-yl)carbamoyl) oxirane-2-carboxylate (**11e**)

**11e** was synthesized by starting from **8e**, 4-phenyl-1-butylamine, as a white powder (45%). $[\alpha]_D$ +45.6 (c = 1.00, MeOH). $^1$H-NMR (DMSO, 400 MHz) δ: 1.17–1.23 (3H, m), 1.35–1.51 (4H, m), 1.74–1.88 (2H, m), 1.99 (3H, s), 2.36-2.40 (2H, m), 2.53 (2H, t, J= 7Hz), 2.96–3.09 (2H, m), 3.59 (1H, d, J= 2Hz), 3.69(1H, d, J= 2Hz), 4.12–4.17 (2H, m), 4.27–4.29 (1H, m), 7.10–7.27 (5H, m), 8.04–8.07 (1H, t, J= 7Hz), 8.63 (1H, d, J= 2Hz). $^{13}$C-NMR (DMSO) δ: 170.2, 167.2, 164.8, 141.6, 128.2, 128.2, 128.1, 128.1, 125.6, 61.5, 52.9, 52.1, 51.2, 38.2, 34.7, 31.8, 31.5, 30.7, 29.9, 14.6, 13.8; ESI-MS: *m/z* = 423 [M+H]$^+$ 445 [M+Na]$^+$. HRMS calcd for $C_{21}H_{30}O_5N_2S$ [M+Na]$^+$: 445.1765, Found: 445.1766.

2-ethyl 3-(4-nitrophenyl) (2*S*,3*S*)- oxirane-2,3-dicarboxylate (**12**)

To synthesize **12**, a solution of **6** (0.0625 mol; 10.00 g) and *p*-nitrophenol (0.0625 mol; 8.69 g) in AcOEt (40 mL) was added dropwise to *N,N'*-dicyclohexylcarbodiimide (0.0625 mol; 12.88 g) in 60 mL of AcOEt at 4–5 °C. The reaction mixture was stirred at 4–5 °C for 3 h, then allowed to warm to room temperature, and stirred for 1 h. The reaction mixture **12**. Recrystallization from AcOEt-*n*-hexane yielded 11.58 g of **12** (0.0412 mol; 66%) as yellow needles. $[\alpha]_D$ +114.8 (c = 1.00, AcOEt). $^1$H-NMR (DMSO, 400 MHz) δ: 1.26 (3H, t, J= 8Hz), 4.06 (1H, d, J= 2Hz), 4.08 (1H, d, J= 2Hz), 4.24 (2H, q, J= 8Hz), 7.58 (2H, d, J= 8Hz), 8.36 (2H, d, J= 8Hz). ESI-MS: *m/z* = 282 [M+H]$^+$.

Ethyl (2*S*,3*S*)-3-(((*R*)-3-(benzylthio)-1-oxo-1-(pentylamino)propan-2-yl)carbamoyl)oxirane-2-carboxylate (**17a**)

To synthesize **15a**, a 50-mL solution of *N,N′*-dicyclohexylcarbodiimide (0.05 mol; 10.32 g) in AcOEt was added dropwise to a round-bottom flask containing a mixture of **13**, *N*-Boc-*S*-benzyl-L-cysteine (0.05 mol; 15.55 g), **14a** *n*-amylamine (0.05 mol; 4.35 g), and 1-hydroxybenzotriazole (0.05 mol; 6.76 g) in 60 mL of AcOEt. After stirring at 5 °C for 1.5 h, the solution was then warmed to room temperature and stirred for an additional 2.5 h. The reaction mixture was filtered and washed with AcOEt. The combined organic layers were washed with 5% HCl, brine, and saturated $NaHCO_3$ solution and brine, and dried over $MgSO_4$. After filtration, removal of the solvent yielded 17.10 g of **15a** (0.045 mol; 90%) as a colorless solid. It was used in the next step without further purification.

To synthesize **16a**, 65 mL of 10% HCl-AcOEt (v/v) was added to 65 mL of a stirred solution of **15a** (0.045 mol; 17.10 g) in AcOEt; the mixture was stirred for 2.5 h at room temperature. The solution was concentrated under vacuum. It was then diluted with water and extracted with AcOEt. The aqueous phase was alkalified with 25% NaOH (pH > 10) and washed with AcOEt. The organic layers were combined, washed with brine, and dried over $MgSO_4$. The AcOEt was evaporated under vacuum, yielding 10.32 g of **16a** (0.037 mol; 82%) as a white oil.

To synthesize **17a**, **12** (0.035 mol; 9.83 g) was dissolved in AcOEt, and an equal amount of **16a** (0.035 mol; 9.77 g) in 50 mL of AcOEt was added dropwise at room temperature over 5 min. The mixture was then stirred at room temperature for 4 h. The reaction mixture was filtered and washed with AcOEt. The combined organic layers were washed with 100 mL of 2% NaOH, 100 mL of brine, 100 mL of 5% HCl (v/v), and 100 mL of brine, successively, and then dried over $MgSO_4$. After filtration, removal of the organic solvent under vacuum yielded a white powder. The crude **17a** was finally purified by silica column

chromatography (0.020 mol; 8.84 g; 58%). $^{1}$H-NMR (DMSO, 400 MHz) δ: 0.81 (3H, t, J= 7Hz), 1.17–1.37 (9H, m), 2.51–2.68 (2H, m), 2.94–3.09 (2H, m), 3.56–3.74 (4H, m), 4.15 (2H, m), 4.49 (1H, q, J= 7Hz), 7.17–7.27 (5H, m), 8.20–8.23 (1H, t, J= 7Hz), 8.74 (1H, d, J= 8Hz). $^{13}$C-NMR (DMSO) δ: 169.2, 167.2, 164.9, 138.3, 129.0, 129.0, 128.4, 128.4, 126.9, 61.6, 52.9, 52.0, 51.3, 38.6, 34.9, 33.0, 28.6, 28.5, 21.9, 14.0, 13.9; ESI-MS: *m/z* = 423 $[M+H]^+$ 445 $[M+Na]^+$ 867 $[2M+Na]^+$. HRMS calcd for $C_{21}H_{30}O_5N_2S$ $[M+Na]^+$: 445.1767, Found: 445.1765.

Ethyl (2*S*,3*S*)-3-(((2*R*)-3-(benzylthio)-1-(*sec*-butylamino)-1-oxopropan-2-yl)carbamoyl)oxirane-2-carboxylate (**17b**)

Similarly, **17b** was synthesized by starting from **14b**, *sec*-butylamine, as a white powder (65%). $[\alpha]_D$ +15.9 (c = 1.00, MeOH). $^{1}$H-NMR (DMSO, 400 MHz) δ: 0.76 (3H, t, J= 7Hz), 0.99 (3H, d, J= 7Hz), 1.15–1.39 (5H, m), 2.50–2.68 (2H, m), 3.56–3.72 (4H, m), 3.96–4.16 (3H, m), 4.50 (1H, q, J= 7Hz), 7.17–7.33 (5H, m), 8.05 (1H, d, J= 7Hz), 8.70 (1H, d, J= 8Hz). $^{13}$C-NMR (DMSO) δ: 169.2, 167.2, 164.8, 138.3, 129.0, 129.0, 128.4, 128.4, 126.9, 61.6, 52.9, 52.0, 51.3, 38.6, 34.9, 33.3, 28.8, 20.3, 13.9, 10.5; ESI-MS: *m/z* = 409 $[M+H]^+$ 431 $[M+Na]^+$. HRMS calcd for $C_{20}H_{28}O_5N_2S$ $[M+Na]^+$: 431.1611, Found: 431.1610.

Ethyl (2*S*,3*S*)-3-(((*R*)-3-(benzylthio)-1-((3-fluorophenethyl)amino)-1-oxopropan-2-yl)carbamoyl)oxirane-2-carboxylate (**17c**).

**17c** was synthesized by starting from **14c**, 3-fluorophenethylamine, as a white powder (50%). $[\alpha]_D$ +19.2 (c = 1.00, MeOH). $^{1}$H-NMR (DMSO, 400 MHz) δ: 1.15–1.21 (3H, t, J= 7Hz), 2.60–2.72 (4H, m), 3.56–3.71 (6H, m), 3.96–4.19 (2H, m), 4.46 (1H, q, J= 7Hz), 6.98 (3H, m), 7.17–7.27 (6H, m), 8.30 (1H, t, J= 7Hz), 8.70 (1H, d, J= 8Hz).

$^{13}$C-NMR (DMSO) δ: 169.2, 167.2, 164.8, 163.5, 142.1, 138.5, 130.9, 129.4, 129.4, 128.9, 128.9, 127.3, 125.2, 115.2, 113.7, 61.1, 53.4, 52.0, 51.5, 35.5, 35.0, 33.8, 33.2, 13.9; ESI-MS: *m/z* = 475[M+H]$^+$ 497 [M+Na]$^+$. HRMS calcd for $C_{24}H_{27}O_5N_2SF$ [M+Na]$^+$: 497.1514, Found: 497.1515.

Ethyl (2*S*,3*S*)-3-(((*R*)-3-(benzylthio)-1-oxo-1-((4-phenylbutyl)amino)propan-2-yl)carbamoyl)oxirane-2-carboxylate (**17d**).

**17d** was synthesized by starting from **14d**, 4-phenyl-1-butylamine, as a yellow powder (41%). $^1$H-NMR (DMSO, 400 MHz) δ: 1.15–1.24 (5H, m), 1.35–1.55 (4H, m), 2.54–2.69 (2H, m), 3.06–3.15 (2H, m), 3.61–3.75 (4H, m), 4.00–4.19 (2H, m), 4.53 (1H, q, J= 7Hz), 7.14–7.32 (10H, m), 8.25–8.28 (1H, t, J= 7Hz), 8.76 (1H, d, J= 8Hz). $^{13}$C-NMR (DMSO) δ: 169.2, 167.2, 164.8, 142.1, 138.2, 128.9, 128.9, 128.8, 128.8, 128.3, 128.3, 128.2, 128.2, 126.8, 125.6, 61.5, 52.9, 52.0, 51.2, 38.3, 34.9, 32.3, 31.9, 30.7, 28.2, 13.8; ESI-MS: *m/z* = 485 [M+H]$^+$ 507 [M+Na]$^+$. HRMS calcd for $C_{26}H_{32}O_5N_2S$ [M+Na]$^+$: 507.1922, Found: 507.1921.

Pharmacology

The inhibitor potency assays were performed using inhibitor screening kits (Biovision, USA, San Francisco, cat#: K150-100, K147-100, K161-100, and K149-100). A total of 2 μL of 2× enzyme solution (cathepsin K, cathepsin B, cathepsin L, and cathepsin S) was added to each well of a 384-well plate. Next, 2 μL of 10× compound (**1**, **2**, **11a–11e**, **17a–17e**; **1** and **2** were purchased from Selleck Company) solution at final compound concentrations of 100, 33.3, 11.1, 3.7, 1.23, 0.41, 0.14, 0.046, 0.015, 005, and 0 μM in DMSO (two replicates for each dose) was added. After centrifugation (1000 rpm) for 1 min and incubation for 15 min at 25 °C, 8 μL of 2.5× substrate solution (substrates were provided in the kits, and the

concentrations used were optimized as recommended in the kit manual), a total of 250 μM/L Ac-LR-AFC for CTSK, 250 μM/L Ac-RR-AFC for CTSB, 125 μM/L Ac-FR-AFC for CTSL, and 250 μM/L Z-VVR-AFC for CTSS) were added and centrifuged (1000 rpm) for 1 min to mix. Finally, the fluorescence signal (excitation: 400 nm; emission: 505 nm) was measured by an Enspire instrument in kinetics mode at 37 °C for 120 min.

The data (duplicate measurements) were fitted to a sigmoidal dose-response curve with a variable slope. The $IC_{50}$ concentrations were calculated from the curve. The relative inhibition was determined by the following equation: Relative inhibition % = (Slope of EC – Slope of S)/(Slope of EC – Slope of IC) × 100, where EC indicates enzyme control, S indicates sample, and IC indicates inhibitor control. Commercially available E64d (**1**) and CA-074 (**2**) were assayed as controls.

**Results and Discussion**

Based on the previously demonstrated specificity of the general cysteine protease inhibitor E64 [9], we set out to synthesize a series of its analogs by incorporation of structural elements at both the $R^1$ and $R^2$ positions in order to further investigate how these structures modulate the specificity of the inhibitor scaffold and to understand the structure-activity relationships of these derivatives as cathepsin B inhibitors. The Chart for synthesizing the new series of epoxysuccinyl-peptide derivatives (**11a–11e**) is shown in Chart **1**. First, crude **4** was obtained by reacting **3** ((+/-)-*trans*-epoxysuccinic acid) with L-arginine in MeOH-water solution (4:1) at room temperature. The product was then recrystallized from MeOH-water (2:1) solution. Subsequently, reacting it with 95% $H_2SO_4$ in ethanol afforded the corresponding diethyl ester (**5**) in 80% yield. **6** was obtained via the reaction of **5** with potassium hydroxide in ethanol and stored under a nitrogen atmosphere at 4–6 °C.

In parallel, an array of **10a–e** was synthesized via a two-step route. It started with the reaction between commercially available **7** and **8a–e** in the presence of *N,N′*-dicyclohexylcarbodiimide, 1-hydroxybenzotriazole, and AcOEt. By this simple acid-amine reaction, different structures were introduced to the $R_2$ position to form **9a–e**. Next, the butyloxycarbonyl protecting group (Boc) was removed under strongly acidic conditions to release the unprotected amine group to react with **6** and eventually afford **11a** (42% yield). **11b–e** were synthesized in a similar manner.

In addition, we evaluated the importance of the $R^1$ diversity site by synthesizing a library of compounds in which benzyl(methyl) sulfane substituted ethyl(methyl) sulfane at the $R^1$ position of the above-mentioned E64 inhibitor analog. In this case, commercially available **13**, *N*-Boc-*S*-benzyl-L-cysteine, was used as a starting material to first react with *n*-amylamine (**14**), followed with deprotection of the amino group to afford **16** (Chart **2**). However, a different route was used to synthesize the target **17**. Instead of directly reacting **6** with **16** (a similar route as shown in Chart **1**), **6** was first reacted with *p*-nitrophenol to convert the acid group to an active ester. The resultant **12** was then reacted with **16** to afford a group of **17** with different substitutents at $R^2$. This intermediate active ester method successfully increased the yield of the final products and eliminated the byproducts observed when using the direct acid method shown in Chart **1**.

In order to evaluate the inhibitory activities and to study the associated structure-activity relationships of the newly synthesized compounds as cathepsin B inhibitors, a pharmacological study was carried out using conventional inhibitor potency assays, and the $IC_{50}$ concentrations were calculated by the sigmoidal dose-response curve method to determine how much of each compound is needed to effectively inhibit cathepsin B. As shown in Table **1**, ethyl(methyl) sulfane-substituted epoxysuccinyl-peptide derivatives **11a–e** appeared to be highly selective inhibitors of cathepsin B. Since cathepsins K, L, and S are

mostly related to cathepsin B in the papain family [14, 15], it1is very critical that effective cathepsin B inhibitors have a low inhibitory activity against them. Relatively low affinities to cathepsins K, L, and S were observed. As compared with **1** and **2** (commercially available E64d and CA-074, respectively), **11a** and **11b** not only demonstrated good inhibitory activities (low cathepsin B $IC_{50}$ values) but also great selectivities, with a much larger ratio of cathepsin B $IC_{50}$ vs. cathepsin X (X: K, L, and S) $IC_{50}$. Thus, these two specifically designed compounds demonstrated the highest selectivities to cathepsin B.

The observed difference in the inhibitory performance of **11a–e** was further linked to their molecular structures at the $R_2$ position. The hexyl substituent at $R^2$ of **11a** showed the strongest inhibitory activity against cathepsin B ($IC_{50}$ = 4.27 ± 1.51 nM) among all tested compounds. By substituting it with the 1-ethyl propyl group, the overall inhibition and selectivity of **11d** to cathepsin B significantly decreased (cathepsin B $IC_{50}$ = 74.27 ± 8.18 nM, that is 22-fold, 129-fold, and 193-fold lower than the $IC_{50}$ for cathepsins K, L, and S, respectively), suggesting that a highly flexible linear hexyl chain is critical for cathepsin B inhibition, compared to a branched segment even with a similar total carbon number (C6 vs. C5). Decent potent inhibition and selectivity for cathepsin B was observed for **11b** (cathepsin B $IC_{50}$ = 7.16 ± 2.26 nM, that is 116-fold, 210-fold, and 524-fold lower than the $IC_{50}$ for cathepsins K, L, and S, respectively) as well. However, the effect of the alkyl chain length to link the benzyl group at the $R^2$ position is complicated in this case. Simply replacing the propyl group in **11b** with a butyl group caused a significant drop in both the inhibitory activity and selectivity of **11e**. The addition of a fluorine electron affinity unit on the benzene ring (**11c**) failed to show much improvement on overall inhibitory performance. Based on this observation, it is very important to maintain a flexible alkyl chain at $R^2$ (C6 here) to benefit the inhibitory activity. Adding a bulky segment such as benzene at the end of the linear chain seems to deleteriously affect its inhibitory performance.

Similarly, we also evaluated the performance of compounds with benzyl(methyl) sulfane substituted at the $R^1$ position. From the preliminary results shown in Table **1**, little cathepsin B inhibitory activity was observed on all studied **17** ($IC_{50}$ > 1000 nM). It is hypothesized that the hydrophobic benzyl group prevented the formation of effective hydrogen-bond interactions between the inhibitor molecule and the two histidine residues in the occluding loop structure of cathepsin B [12].

## Conclusions

We synthesized a library of epoxysuccinyl-peptide derivatives with various substituents at the $R^1$ or $R^2$ positions of the model molecule E64d. As compared to the benyl(methyl) sulfane at $R^1$, compounds with an ethyl(methyl) sulfane substituent showed enhanced inhibitory activity and selectivity against cathepsin B. In particular, when a linear hexyl linkage group was incorporated at the $R^2$ position, the resultant **11a** exceeded the performance of the benchmark E64d and CA-074 compounds as cathepsin B inhibitors. However, the molecules with a benzyl(methyl) sulfane substituent at the $R^1$ position failed to demonstrate much cathepsin B inhibitory activity. In future work, besides continuing to study the effect of the chain length of the linear alkyl group at the $R^2$ position, we will also focus on studying the size, chain length, and hydrophobicity of substituents at the $R^1$ and $R^2$ positions to understand the structure-activity relationship of this model molecule as a cathepsin B inhibitor.

## Conflict of Interest

The authors declare no conflict of interest

## Acknowledgments

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**Figure legends**

Figure 1. Chemical structures of the cathepsin B inhibitors E64d and CA-074.

Figure 2. Design of compound as a cathepsin B inhibitor.

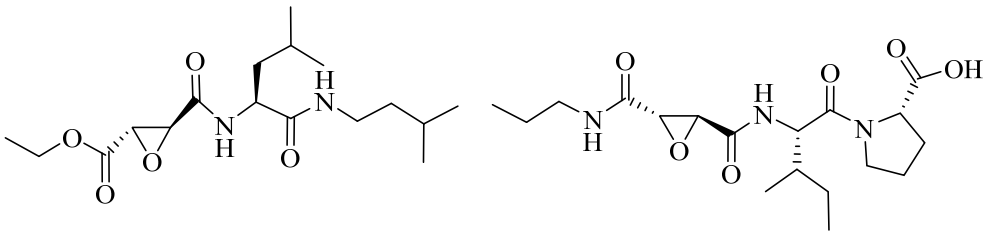
Chart 1. Chart for the preparation of derivatives **11a–11e**. Reagents and conditions: (a) L-arginine, MeOH, r.t. overnight, recrystallization in MeOH-water (2:1); (b) 95% $H_2SO_4$, EtOH, 4.5 h at r.t.; (c) 85% KOH, EtOH, 4 h at 4 °C; (d) $NH_2$-$R_2$ **8a–e**, HOBt, DCC, AcOEt, 1.5 h at 5 °C then 2.5 h at r.t.; (e) 10% HCl-AcOEt, 2.5 h at r.t.; (f) 6, DCC, HOBt, AcOEt, 3 h at 5 °C then 15 h at r.t.

Chart 2. Chart for the preparation of derivatives **17a–d**. Reagents and conditions: (a) *p*-nitrophenol, DCC, AcOEt, 3 h at 5 °C then 1 h at r.t.; (b) $NH_2$-$R_2$ **14a–e**, HOBt, DCC, AcOEt, 5 °C 1.5 h then r.t., 2.5 h; (c) 10% HCl, AcOEt, r.t., 2.5 h; (d) **12**, AcOEt, r.t., 4 h.

Table 1. Cathepsin B inhibition data for compounds **11a–e** and **17a–d**.

| Compound ID | Cathepsin B $IC_{50}$ (nM) | Cathepsin B $IC_{50}$ vs. | | |
|---|---|---|---|---|
| | | Cathepsin K $IC_{50}$ (fold) | Cathepsin L $IC_{50}$ (fold) | Cathepsin S $IC_{50}$ (fold) |
| **1** | 12.03 ± 2.03 | 39 | 15 | 55 |
| **2** | 6.61 ± 0.61 | 38 | 164 | 510 |
| **11a** | 4.27 ± 1.51 | 181 | 278 | 497 |
| **11b** | 7.16 ± 2.26 | 116 | 210 | 524 |
| **11c** | 24.09 ± 2.59 | 21 | 115 | 167 |
| **11d** | 74.27 ± 8.18 | 22 | 129 | 193 |
| **11e** | 23.68 ± 4.78 | 28 | 185 | 149 |
| **17a** | >1000 | -- | -- | -- |
| **17b** | >1000 | -- | -- | -- |
| **17c** | >1000 | -- | -- | -- |
| **17d** | >1000 | -- | -- | -- |

Figure 1



**1 E64d** **2 CA-074**

Figure 2

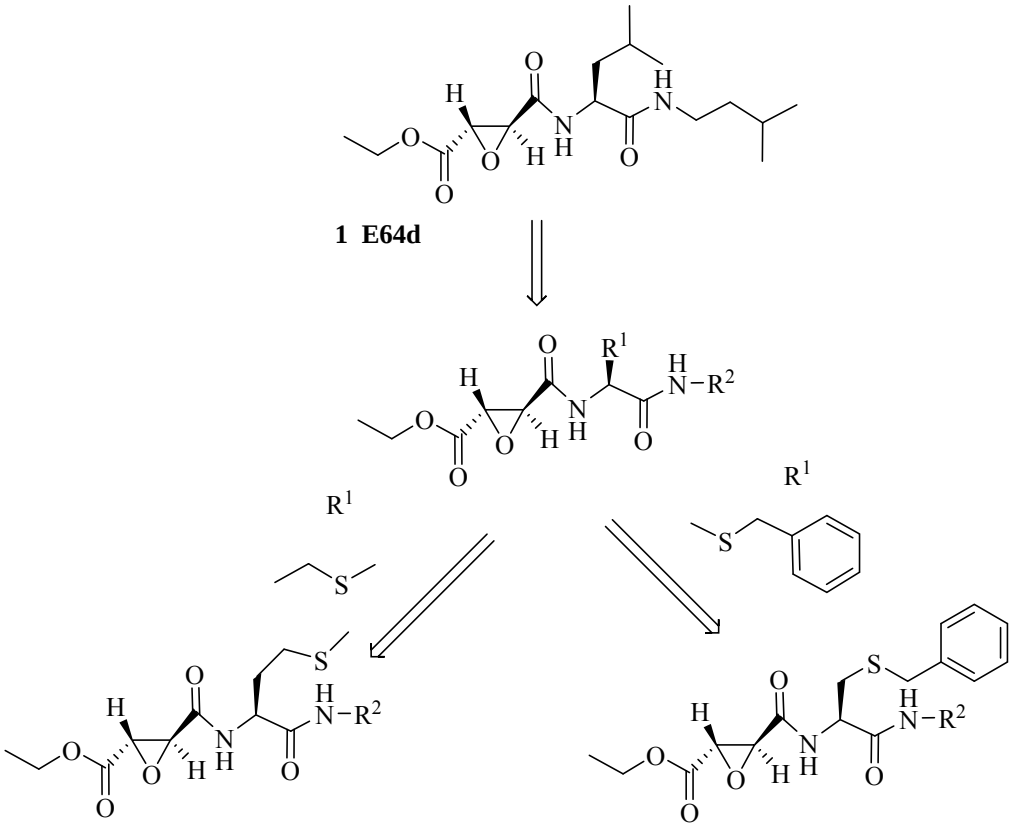
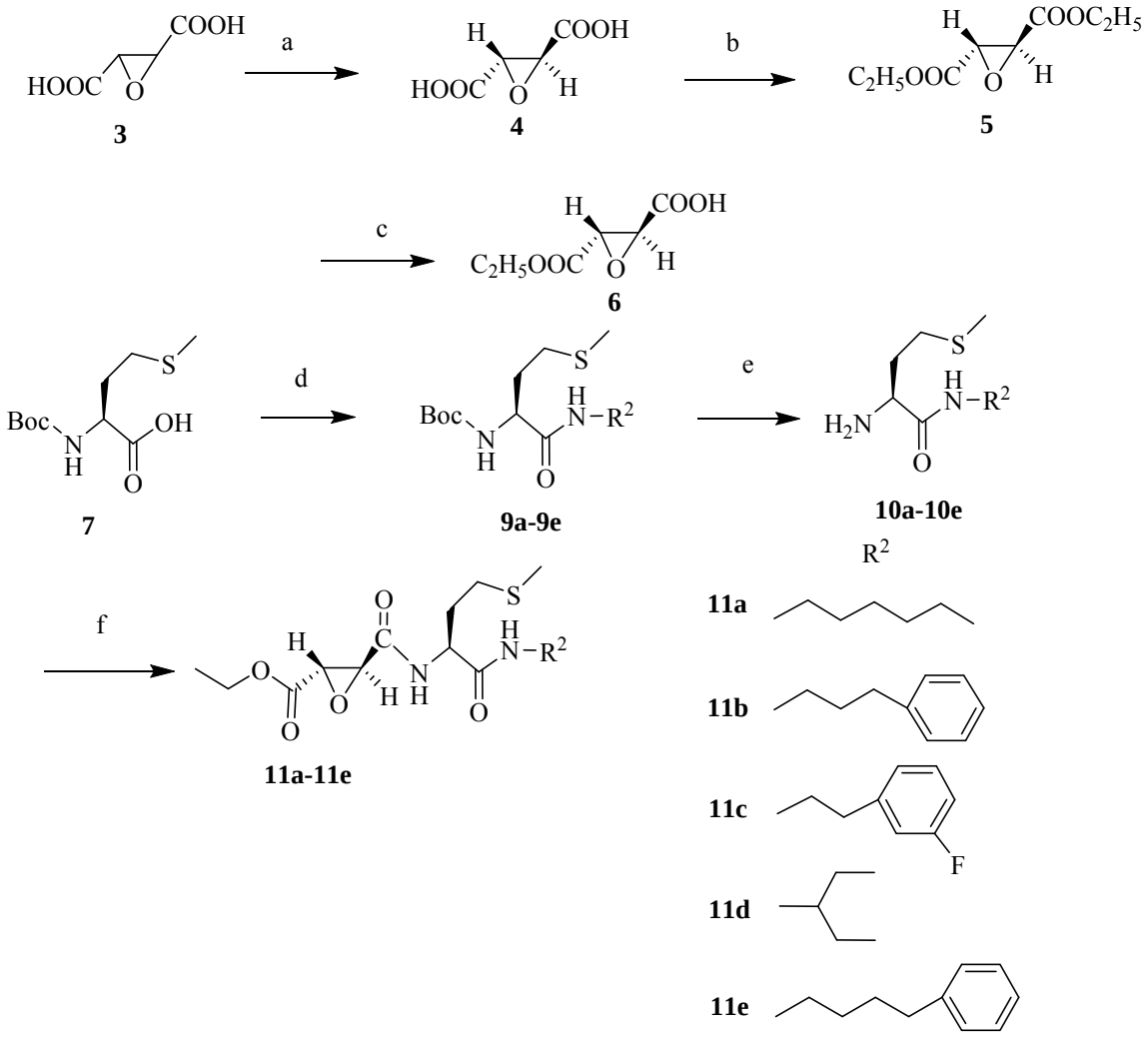


Chart 1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H
C
N
H
O
S
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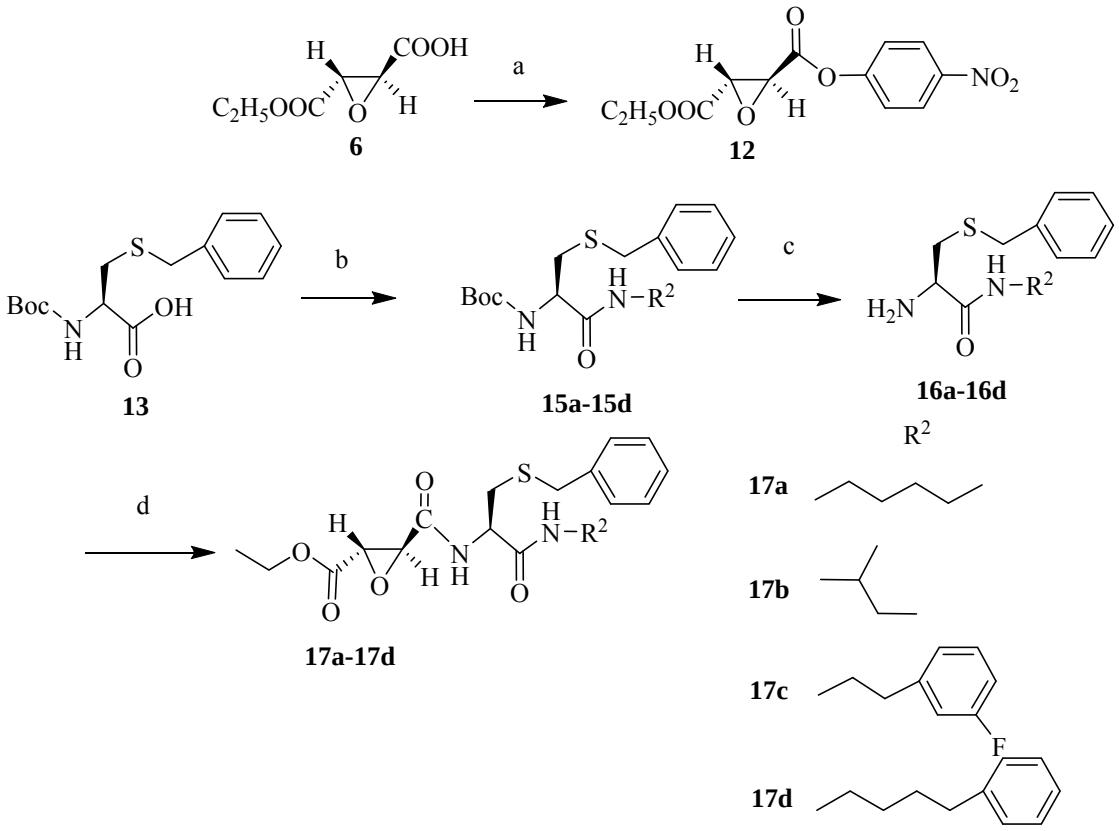
11b
11c
F
11d
11e

Chart **2**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Boc
N
H
OH
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13
b
S
H
Boc
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