

Solid-Phase Methods for the Preparation of Epoxysuccinate-Based Inhibitors of Cysteine Proteases

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The CA clan papain family is one of the largest and best studied subfamilies of the cysteine proteases. In addition to their association with physiological processes such as antigen presentation,¹ it has become clear that these enzymes are involved in several pathological processes including rheumatoid arthritis,² Alzheimer's disease,³ cancer invasion and metastasis,⁴ and several parasitic diseases.⁵ Selective inhibition of papain cysteine proteases will aid in our understanding of their role in these human diseases. More importantly, recent studies using papain family selective epoxysuccinyl inhibitors in mouse models of cancer suggest they may represent a viable new class of anticancer chemotherapeutic agents.⁶ Currently, one such epoxysuccinate inhibitor is in preclinical trials at the National Cancer Institute. Thus, methods allowing the rapid synthesis of libraries of diverse epoxysuccinates that contain reduced peptide character will be critical for the identification of optimal lead compounds for use in human clinical trials.

The natural product E-64⁷ (**1**, Figure 1) and its closely related analog JPM-565⁸ (**2**) are epoxysuccinyl-based covalent inhibitors of papain-like cysteine proteases. Their broad-spectrum activity against this family of enzymes is derived from the leucine residue that mimics the P2 position of a substrate and interacts with the hydrophobic S2 pocket of most papain-like proteases.

The ethyl ester of **2**, JPM-OEt (**3**), has been used in vivo studies using a mouse model for pancreatic cancer.⁶ Treatment with JPM-OEt resulted in a significant reduction in angiogenesis, tumor volume, and tumor invasiveness. Combined with overall low toxicity, these results support the development of this class of inhibitors as anticancer chemotherapeutics. However, the very short half-life of JPM-OEt in vivo and its overall limited bioavailability makes it less suitable as a drug candidate.

We recently reported a solid-phase synthesis route that allows the incorporation of variable peptide elements on both sides of the epoxide function.⁹ Synthesis of a number of "double-headed" epoxides using this method confirmed the importance of the interactions of inhibitors with regions of the active site on both sides of the reactive cysteine nucleophile. However, the current synthetic methods are only able to produce compounds that are highly peptidic in nature

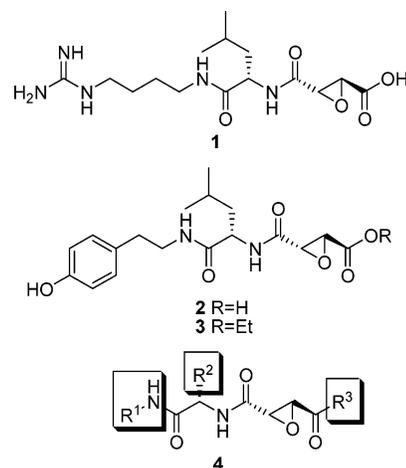


Figure 1. Structures of natural product E-64, its synthesized analogs, JPM-565 and JPM-OEt, and the general scaffold **4**.

and therefore unlikely to have optimal pharmacological properties. Furthermore, the incorporation of diversity elements is limited by the need for suitably protected amino acid building blocks for attachment to the resin.

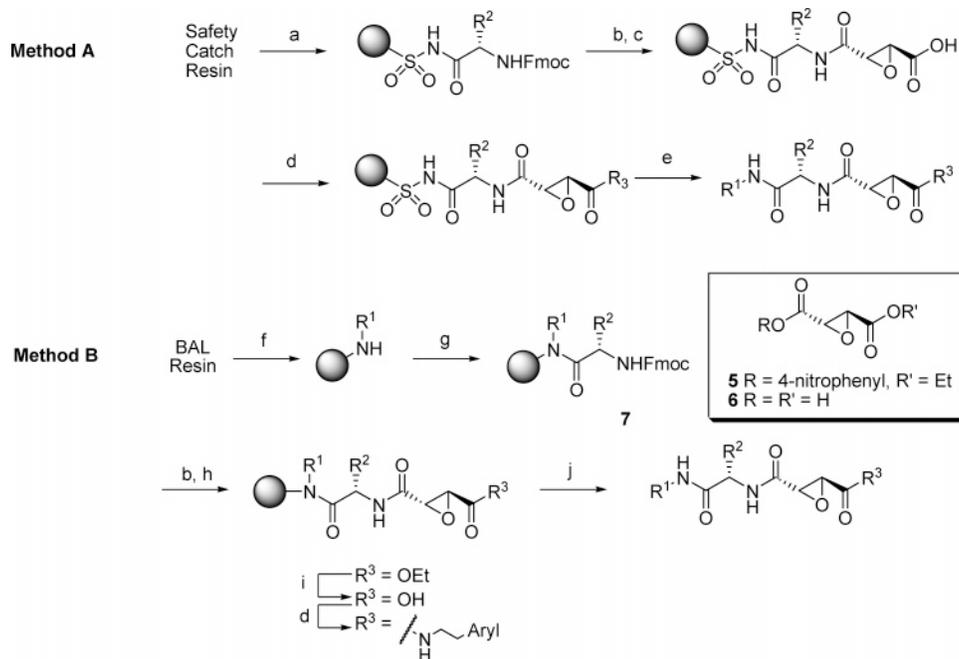
We reasoned that the incorporation of non-natural elements would be an advantageous strategy for the design of a cathepsin inhibitor because it would allow for the introduction of a larger diversity of R groups within the inhibitor scaffold (**4**, Figure 1). In addition, the reduction of the peptide character would significantly increase the druglike properties of the resulting compounds.

The use of diverse amines to generate the R¹ element on the inhibitor scaffold (see Figure 1) precludes the use of the standard Rink linkage to the resin. To address this issue, we investigated resins that have been applied to make C-terminal-modified peptides.¹⁰

In our first approach, a safety-catch resin was loaded with the critical P2 amino acid using reported procedures (Scheme 1, method A).¹¹ Next, we introduced the epoxysuccinate warhead using the activated nitrophenyl ester **5**, followed by hydrolysis of the ethyl ester to introduce a R³ group at the prime side of the molecule. However, treatment of the resin under saponifying conditions, as previously described,⁹ resulted in premature cleavage from the solid support and therefore proved incompatible with the safety-catch resin. To overcome this problem, we coupled epoxysuccinate **6**,¹² having two free acid groups, with PyBOP/DIEA. The resulting immobilized acid could then be directly extended by coupling of the R³ amine using a standard PyBOP/DIEA-mediated coupling.⁹ Activation of the sulfonamide function by iodoacetonitrile and the subsequent release from the resin using an excess of the R¹-NH₂ yielded the desired compounds in a 12–20% yield after HPLC purification (based on original resin load reported by vendor; see Table 1).

Although the safety-catch resin provides the desired products in reasonably good yields, the presence of an excess of the amine during cleavage from the resin results in a

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Scheme 1. Two Strategies Toward Epoxysuccinate-Based CA Clan Inhibitors Using Safety-Catch and BAL Resins.^a

^a Reagents and conditions: (a) Fmoc-amino acid (3 equiv), DIEA (6 equiv), PyBOP (3 equiv), DCM, $-20\text{ }^{\circ}\text{C}$; (b) DMF/piperidine 4/1; (c) **6** (3 equiv), PyBOP (3 equiv), DIEA (6 equiv), DMF; (d) R³-NH₂ (3 equiv), DIEA (6 equiv), PyBOP (3 equiv), DMF; (e) iodoacetone (20 equiv), R¹-NH₂ (5 equiv), NMP; (f) R¹-NH₂ (10 equiv), NaBH₃CN (10 equiv), DMF; (g) Fmoc-amino acid (10 equiv), HATU (10 equiv), DIEA (20 equiv), DMF; (h) **5** (3 equiv), DMF; (i) 1 M KOH, THF/EtOH 3/1; (j) 95% TFA/2.5% TIS/2.5% H₂O.

Table 1. Representative Compounds Synthesized Using the Described Methods A and B

| AMS# | R ¹ | R ² | R ³ | Yield | Method |
|------|----------------|---|----------------|-------|--------|
| 17 | | CH ₂ CH ₂ (CH ₃) ₂ | | 12% | A |
| 20 | | CH ₂ CH ₂ (CH ₃) ₂ | | 13% | A |
| 20 | | CH ₂ CH ₂ (CH ₃) ₂ | | 12% | B |
| 27 | | CH ₂ CH ₂ (CH ₃) ₂ | | 20% | A |
| 33 | | CH ₂ CH ₂ CH ₃ | | 14% | B |
| 34 | | CH ₂ CH ₂ CH ₃ | OEt | 42% | B |
| 35 | | CH ₂ CH ₂ CH ₃ | OH | 37% | B |

significant amount of unwanted side-products. Moreover, we observed ring opening of the epoxide by the amine if the reaction mixture after cleavage was concentrated without the addition of acid to quench the nucleophilic amine.

As an alternative, we turned our attention to the BAL (backbone-amide linker) resin, which links C-terminal-modified peptide derivatives to the resin through their amide bond (Scheme 1, method B). The BAL resin uses an aldehyde group that is transformed into a Schiff base upon treatment with an amine. After reduction, the resulting secondary amine can be used as a handle to perform solid-phase peptide

elongation. For this synthetic method, the desired R¹ amines were loaded onto the BAL resin using sodium cyanoborohydride, as previously reported.¹³ Next, the P2 amino acid was coupled to the resin-bound secondary amine in high yields using HATU/DIEA in DMF. After Fmoc deprotection, activated epoxysuccinate **5** was coupled to the free amine, and the ethyl ester was hydrolyzed with KOH as previously described.⁹ The resulting free acid was then used for coupling of the R³ amine using standard PyBOP-coupling methods. The products were cleaved from the resin with TFA and purified by HPLC (12–42% yield, relative to resin **7**; see

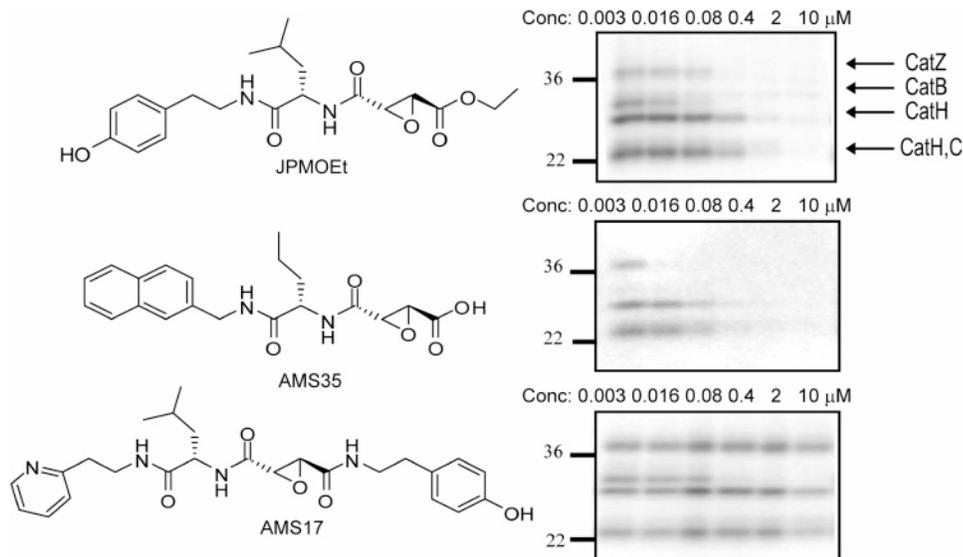


Figure 2. Screening of the representative inhibitors for selectivity and potency against endogenous cathepsin cysteine proteases in rat liver extracts. Compounds (shown at left) were added to rat liver extracts at the indicated final concentrations. After 20 min of pretreatment, the residual activity of cathepsins Z, B, H, and C was measured by labeling with a general radiolabeled activity-based probe ^{125}I -DCG-04. Samples were analyzed by SDS-PAGE and labeled proteases visualized by autoradiography. Loss of labeling indicates binding of the inhibitor.

Table 1). For comparison, AMS20 was synthesized using both methods A and B, which showed similar final yields.

To screen the inhibitors for potency and selectivity in crude protein extracts, we used an established gel-based competition assay.¹⁴ Accordingly, rat liver homogenates were incubated with each of the inhibitors listed in Table 1 over a range of concentrations. The residual protease activity was measured by the addition of the radiolabeled cathepsin probe ^{125}I -DCG-04.¹⁵ Representative data for the most potent and selective inhibitors in the series compared to the parent JPM-OEt are shown in Figure 2. Interestingly, AMS35 was an approximately 5-fold more potent general cathepsin inhibitor in rat-liver lysate than JPM-OEt. Furthermore, AMS17 showed complete inhibition of cathepsin B at 400 nM, leaving the other cathepsins virtually unaffected up to 10 μM.

In conclusion, we have outlined solid-phase methods to synthesize epoxysuccinate-based clan CA inhibitors displaying diverse nonpeptidic groups. Diversification of the scaffold of the epoxysuccinate resulted in cysteine cathepsin inhibitors with improved potency and selectivity properties. The described methods will allow rapid synthesis of diverse inhibitors that can be used to identify new classes of lead compounds for use as cancer chemotherapeutics. It is expected that these will help identify compounds with improved pharmacodynamic properties. Efforts along these lines are currently under investigation and will be published in the future.

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Supporting Information Available. Experimental procedures and spectroscopic data for new compounds. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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