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# A General Solid Phase Method for the Preparation of Diverse Azapeptide Probes Directed Against Cysteine Proteases

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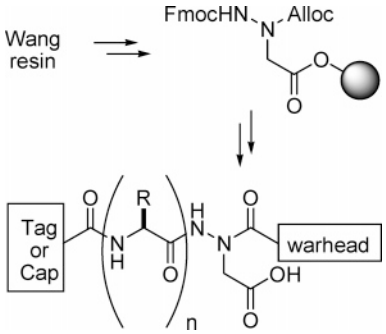
## ABSTRACT




**A solid phase approach is presented for the synthesis of azapeptide inhibitors and activity based probes (ABPs) for cysteine proteases. This synthetic method allows the incorporation of diverse reactive warheads linked to different peptide recognition elements. Application of this method to the synthesis of a series of caspase probes is described.**

Proteases represent one of the largest families of enzymes found in the human genome. Recent efforts to classify proteases has resulted in the assignment of "clans" as a means to delineate groups of related enzymes.[1] Of all the cysteine proteases, clans CA and CD are two of the largest and most well characterized subclasses. The first clan consists of the papain-fold enzymes, which are involved in several pathological processes, such as tumor invasion,[2] arthritis,[3] and numerous parasitic infections.[4] The second clan includes—among others—the caspases, which play a central role in apoptosis.[5]

One of the more promising methods for the study of protease function involves the use of so-called activity based probes (ABPs) that can selectively form a covalent bond with the active site of a protease and concomitantly link the enzyme to a visualization or purification tag.[6] Both clan CA and CD clan cysteine proteases have been targeted by ABPs that contain a range of reactive warhead groups linked to peptide recognition elements carrying tags, such as fluorophores, biotin, or radioactive tracers.[6] However, to develop novel, highly specific probes for application to functional

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proteomic studies, methods are required that allow the efficient synthesis of probes that contain diverse recognition elements.

The covalent epoxysuccinyl scaffold derived from the natural product E-64[7] has been employed by our laboratory to design ABPs for clan CA cysteine proteases (Figure 1).

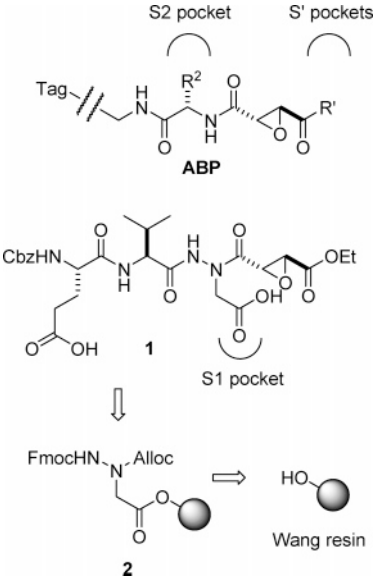


**Figure 1.** A generic structure of an ABP derived from E-64 (**ABP**) relative to the azapeptide inhibitor **1** that can be synthesized from the doubly protected intermediate **2** using solid phase peptide chemistry.

This class of compounds has been used to explore diversity in the P2−P4[8] and prime side[9] functional elements as a means to design probes that target small subsets or individual clan CA proteases.

An intrinsic feature of the epoxysuccinyl scaffold is the lack of a P1 amino acid, the presence of which is pivotal for recognition by clan CD proteases. The caspases, for example, exclusively interact with substrates and inhibitors having an aspartate residue in this position. Therefore, E-64-derived peptidyl epoxysuccinates cannot be used to target clan CD proteases.

Recently, Powers and co-workers reported the synthesis of the so-called azaepoxides, an example of which is depicted in Figure 1.[10] A critical feature of these compounds is the hydrazine moiety that allows display of functional elements that can mimic a P1 amino acid side chain. Furthermore, the use of the aza scaffold allows the orientation of the peptide to be inverted relative to E-64, thus creating inhibitors that more accurately mimic a peptide substrate. Since the azapeptides contain a P1 element, they have been used to develop highly potent inhibitors for clan CD proteases.[10] However, their use as ABPs has not been explored. In addition, the reported synthesis of azaepoxides entails solution phase chemistry with only moderate yields. For example, compound **1** was isolated in approximately 10% yield starting from the corresponding peptide methyl ester.[10c] To systematically vary the peptide part of these inhibitors and improve the synthetic throughput, a solid phase methodology would be desirable.

We here describe the first solid phase approach that allows efficient synthesis of azaepoxides containing both diverse peptide scaffolds as well as reactive electrophilic warheads.

Unlike the peptidyl epoxysuccinates, which can be obtained by standard peptide synthesis methods followed by capping of the amino terminus with the epoxide warhead, the azaepoxides require a different approach due to their opposite backbone polarity. We initially decided to utilize the acetate substituent of a P1 "aza-aspartate" as a handle for attachment to the solid support. This would allow synthesis of a diverse range of compounds containing a P1 aspartic acid element that would target caspase proteases. Retrosynthetic analysis (Figure 1) shows that resin **2** represents a key intermediate, allowing elongation of the peptide on the Fmoc-protected nitrogen and subsequent introduction of the warhead at the nitrogen that was temporarily protected by the Alloc group. This strategy features complete synthesis of the peptide scaffold prior to introduction of the reactive electrophilic group, thereby circumventing potential problems associated with undesired nucleophilic attack of the warhead during peptide synthesis.

Starting from commercially available Wang resin, acylation with bromoacetyl bromide and DIEA in the presence of a catalytic amount of 4-dimethylamino pyridine afforded solid support **3** (Scheme 1). For the substitution of the bromide by Fmoc-hydrazine, the concentration turned out to be of crucial importance for an efficient reaction. Reasonable to high conversions were achieved by treatment of the resin **3** with a 0.4 M Fmoc-hydrazine solution in DMF (Scheme 1).

To allow the introduction of the warhead in the final stage of the synthesis, the secondary amine was protected with an Alloc group. Incubation of **4** with 4 equiv of allyl chloroformate for 1 h yielded desired resin **2** in good efficiency as judged by TFA cleavage of an aliquot of resin. It must be noted that longer reaction times and larger amounts of allyl chloroformate resulted in unwanted carbamoylation of the −NH Fmoc group.

Having the desired hydrazide resin in hand, we applied standard solid phase peptide chemistry using DIC/HOBt as

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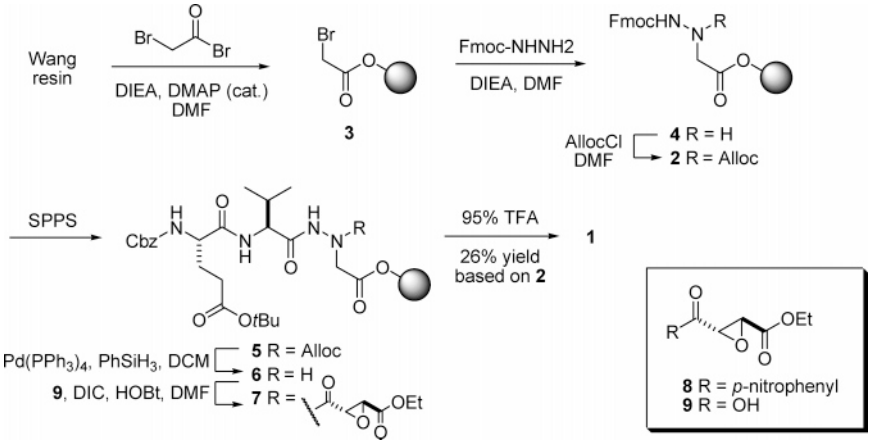
**Scheme 1.** Solid Phase Synthesis of Azaepoxide **1**




activating agents. For efficient coupling to the hydrazine nitrogen, the use of 10 equiv of amino acid at a 0.5 M concentration was required. Other coupling reagents, including PyBOP, HBTU, and DIC/HOAt, did not show an improvement.

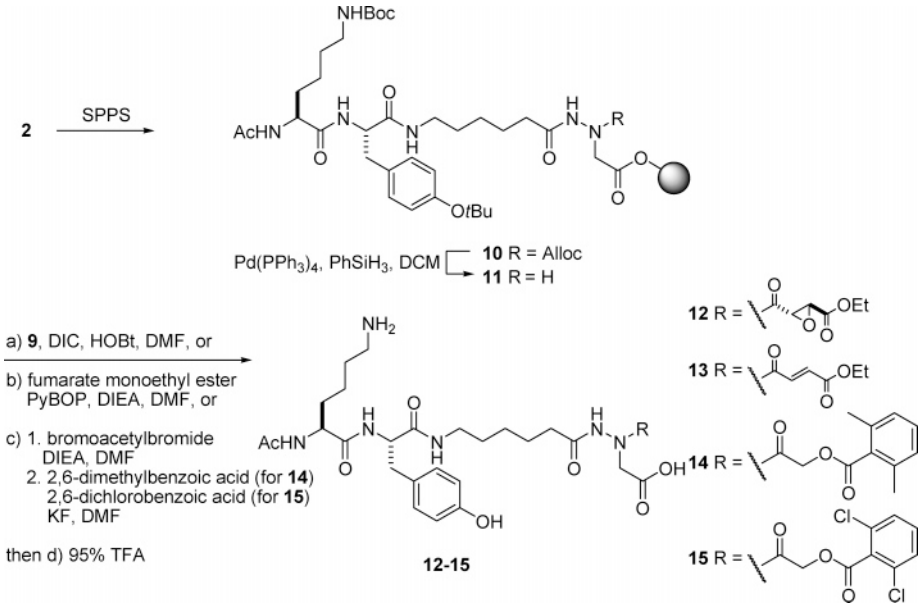
After the amino terminus of the small peptide had been capped with a Cbz group, the Alloc group was deprotected under reductive conditions utilizing $Pd(PPh_3)_4$ as a catalyst. Our standard reagent for installation of the epoxide warhead,[9] the nitrophenyl ester of ethyl epoxysuccinate (**8**), failed to give the desired azaepoxide. However, when we applied the free acid **9** in combination with DIC and HOBt, effective coupling was achieved. Cleavage from the resin and concurrent deprotection of the *tert*-butyl protecting group on the side chain of glutamic acid gave the desired inhibitor **1** in 26% yield after HPLC purification.

Having developed an efficient solid phase method for the azapeptide epoxides, we set out to apply this method to synthesize compounds that contain diverse reactive electrophiles as well as functional groups on the peptide scaffold that facilitate their application as ABPs. Initially, an aminohexanoic acid was incorporated as a spacer to separate the aza-aspartate recognition site from a tyrosine−lysine tagging element (Scheme 2). This dipeptide part of the molecule can be radioactively iodinated at the tyrosine residue or conjugated with a fluorophore or a biotin tag via the free $\epsilon$-amino group on lysine.

Solid phase peptide synthesis and capping of the amino terminus furnished resin **10**. Deprotection of the Alloc and subsequent conjugation to ethyl epoxysuccinate gave compound **12** in high yield (50% based on resin **2**) after cleavage from the resin and HPLC purification.

Since the reactive warhead is installed in the very last step of the solid phase method, it was possible to install different electrophiles on the same general probe scaffold. A recent publication described azapeptide Michael acceptors as ef-

**Scheme 2.** Synthesis of Cysteine Protease ABPs with Different Warheads



fective caspase inhibitors.[11] A probe analogue containing the reported Michael acceptor was easily synthesized from **11** by coupling of fumarate monoethyl ester with PyBOP, and the desired product was isolated in reasonable yield (13%) after HPLC purification.

A novel class of probes was designed based on the acyloxymethyl ketone (AOMK), a warhead that was successfully used by our group for the synthesis of ABPs that target cysteine proteases of both CA and CD clans.[12] Making use of our newly devised solid phase method, the synthesis of an aza counterpart was undertaken. To this end, the secondary amine of resin **11** was equipped with a bromoacetyl group. Next, the bromide was displaced by 2,6-dimethyl- or 2,6-dichlorobenzoic acid under influence of KF.[13] Cleavage from the resin and HPLC purification furnished the desired "aza-AOMKs" **14** and **15** in 34 and 14% yield, respectively.

In summary, a flexible solid phase strategy for production of azapeptidyl inhibitors and ABPs for clan CD cysteine proteases is reported. The utility of this method has been exemplified by the synthesis of the known caspase inhibitor **1** in a superior yield to the reported solution phase approach and the construction of a number of compounds suitable for applications as ABPs. The biochemical application of these probes is currently under investigation and will be reported shortly.

It is expected that the presented approach can be broadened to allow targeting of additional classes of proteases by incorporation of a range of P1 elements on the hydrazine nitrogen. For example, simple exchange of the Wang resin for a Rink resin will lead to an asparagine side chain, which forms a recognition element for the legumain proteases. Current efforts are focused on the design of strategies that will allow incorporation of a variety of P1 side chains through the use of alternate linkers.

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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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