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Solid-Phase Synthesis of Peptide Vinyl Sulfones as Potential Inhibitors and Activity-Based Probes of Cysteine Proteases

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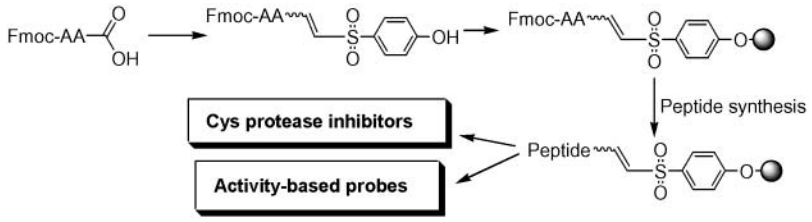
ABSTRACT



Peptide vinyl sulfones were prepared from 2-chlorotrityl resin-bound phenolic amino vinyl sulfones in high yield and purity. This method enables the convenient synthesis of peptide vinyl sulfones having different amino acids at the P_1 position. It also allows efficient synthesis of vinyl sulfone-containing, activity-based probes of cysteine proteases used in a proteomic experiment.

Cysteine proteases are an important class of enzymes involved in the hydrolysis of peptide amide bonds. They play vital roles in numerous physiological processes such as arthritis, osteoporosis, Alzheimer's disease, cancer cell invasion, and apoptosis.[1] Many low-molecular weight inhibitors of cysteine proteases such as epoxysuccinyl derivatives, peptidyl Michael acceptors, (acyloxy)methyl ketones, and halomethyl compounds have been reported.[2] Recently, peptidyl vinyl sulfones and their derivatives were found to inactivate cysteine proteases selectively.[3] They function by mimicking the peptide substrates of cysteine proteases. Upon binding to the active site of the enzyme, the vinyl sulfone moiety of the inhibitor acts as a Michael acceptor and reacts with the nucleophilic cysteine residue located within the active site, resulting in the formation of a covalently linked, irreversible enzyme−vinyl sulfone complex. The advantage of such mechanism-based, covalent reactions has led to their application in mechanistic studies of proteases and proteasomes,[4] protein engineering and modifications,[5] and more recently, activity-based protein profilings.[4,6]

Typically, peptide vinyl sulfones have been prepared by methods based on conventional solution-phase peptide synthesis,[1,3,4a] whereby individual amino acid-containing vinyl sulfones were synthesized, followed by sequential couplings of additional amino acid residues to make up the full-length peptide chain. The whole synthesis was done in solution, making the process inefficient and time-consuming. More recently, a number of solid-phase strategies have been

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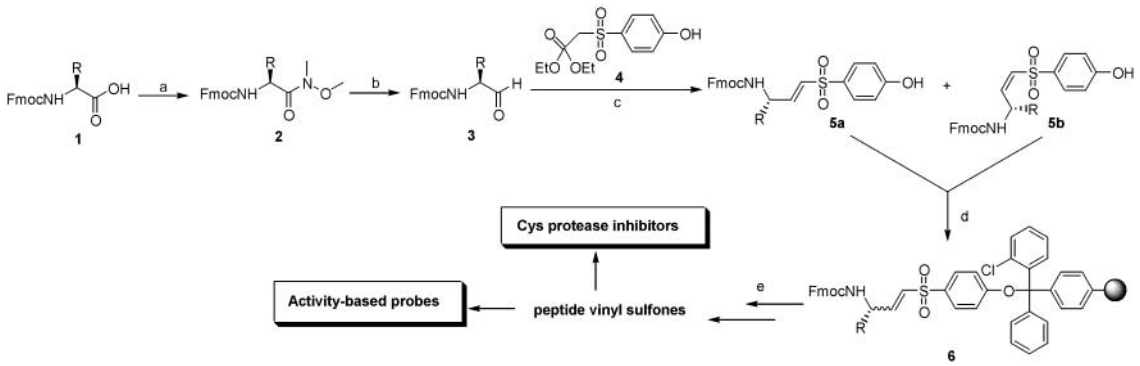
Scheme 1. Solid-Phase Synthesis of Peptide Vinyl Sulfones[a]



[a] Reagents and conditions: (a) *N*,*O*-Dimethylhydroxylamine, DCC/HOBt/DIEA, DMF, 5 h at rt, 80−99%. (b) $LiAlH_4$, THF, 15−20 min at 0 °C, 52−90%. (c) **4** (1 equiv), NaH, THF, 1.5 h at rt, 59−78%. (d) Pyridine, Cs_2CO_3, THF, 12−18 h at rt, 0.43−0.52 mmol/g loading. (e) Fmoc solid-phase peptide synthesis.

developed.[7,4b] By taking advantage of Kenner's safety catch strategy,[8] Overkleeft et al. first synthesized the N-terminal peptide fragment of the vinyl sulfone on a solid support, followed by nucleophilic cleavage/ligation using a desired vinyl sulfone-containing, C-terminal amino acid. Deprotection of the resulting product followed by HPLC purification gave the final peptide vinyl sulfone in 20−40% yield.[7] This method is intrinsically inefficient and low yielding, due to the generation of a fully protected peptide product following the cleavage/ligation step.[9] Consequently, this makes it difficult to synthesize vinyl sulfones having longer peptide chains. Alternatively, Nazif and Bogyo reported a solid-phase method for generating positional-scanning combinatorial libraries of peptide vinyl sulfones.[4c] By attaching a vinyl sulfone-containing aspartic acid onto a Rink amide resin via its side-chain carboxylic acid, these workers were able to generate P_2−P_4 positional-scanning tetrapeptidic vinyl sulfone libraries while holding the P_1 position constant. However, this strategy is limited only to synthesis of peptide vinyl sulfones having carboxyl side chains at the P_1 positions (e.g. Asp and Glu).

Herein, we report a facile yet efficient solid-phase method that may be used for the preparation of peptide vinyl sulfones having any amino acid at the P_1 position. By anchoring the vinyl sulfone-derivatized P_1 amino acid residue onto 2-chlorotrityl resin via the phenolic alcohol moiety of the vinyl sulfone (Scheme 1), this strategy allows the generation of peptide vinyl sulfones from any peptide sequence, either individually or combinatorially, with high yield and efficiency. Furthermore, this strategy may be used for facile synthesis of activity-based probes to specifically target cysteine proteases in a crude proteome mixture.[4,6] With the increasing emphasis in the field of proteomics, this method thus provides a valuable tool for (1) the generation potential inhibitors of cysteine proteases critical for diseases and (2) the identification/profiling of new cysteine proteases in an organism.

Our strategy took advantage of peptide vinyl sulfones containing a phenol group adjacent to the vinyl sulfone moiety. It had been previously shown that having a phenyl or phenolic group (instead of a methyl group) next to the vinyl sulfone not only does not compromise the inhibitory activity of the peptide vinyl sulfone toward its targeting enzyme but, in many cases, actually enhances the potency of the inhibitor.[4b] We reasoned that, by modification of the P_1 amino acid with a phenolic vinyl sulfone, followed by loading the resulting product (e.g., **5** in Scheme 1) onto a suitable solid support via the phenolic alcohol, any peptide vinyl sulfone may be potentially synthesized with high efficiency using conventional solid-phase peptide synthesis. We chose to develop such a strategy on the basis of Fmoc chemistry, as it is the preferred method for solid-phase peptide synthesis. Our first task was to synthesize *N*-fluorenylmethoxycarbonyl (Fmoc)-α-amino aldehydes. They were conveniently synthesized by a well-established, two-step procedure, involving the transformation of *N*-Fmoc-α-amino acid to the corresponding Weinreb amide, followed by reduction with $LiAlH_4$.[10] The *N*-Fmoc-α-amino acids **1** were first coupled with *N*,*O*-dimethylhydroxylamine to give the resulting amides **2** in 80−99% yields, followed by $LiAlH_4$ reduction at 0 °C to give the aldehydes **3**. Aldehydes corresponding to all 20 natural amino acids were synthesized, most of which afforded the desired products in good yields (52−90%; Table 1). Examination of the products showed that most aldehydes were free of racemization and had high purities, except for proline and arginine, the former giving a racemic mixture and the latter forming an intramolecular ring.[11] Subsequent steps were carried out with five randomly

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Table 1. Yield of N−Fmoc-α-amino Aldehydes **3**

3	yield (%)	**3**	yield (%)
Fmoc-Val	52	Fmoc-Gly	89
Fmoc-Asp(tBu)	86	Fmoc-Ala	81
Fmoc-Leu	81	Fmoc-Lys(Boc)	87
Fmoc-Tyr(tBu)	85	Fmoc-Trp(Boc)	64
Fmoc-Glu(tBu)	52	Fmoc-Phe	90
Fmoc-Ser(tBu)	80	Fmoc-Cys(Trt)	89
Fmoc-His(Trt)	67	Fmoc-Asn(Trt)	78
Fmoc-Ile	79	Fmoc-Gln(Trt)	76
Fmoc-Met	80	Fmoc-Thr(tBu)	87

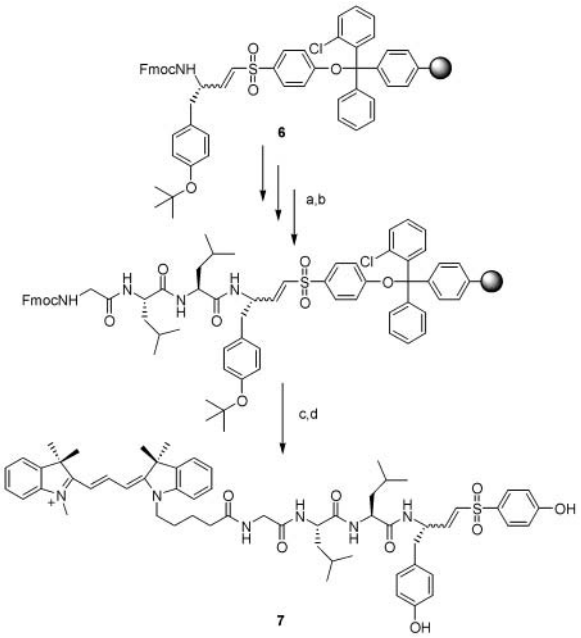
chosen amino aldehydes. *N*-Fmoc-α-amino aldehydes, **3**, were reacted with 4-hydroxy-thiophenyl-methyl-diethylphosponate, **4**, prepared as previously reported,[4b] to give phenolic-*N*-Fmoc-α-amino-vinyl sulfones **5** in a Horner−Emmons condensation reaction. The reaction was carried out smoothly in the presence of NaH at room temperature, generating both the trans isomer (e.g., **5a** in Scheme 1) as the predominantly major product (>90% as judged by ^{1}H NMR and HPLC), and the cis isomer, **5b**, with acceptable yield (59−78% for **5a** + **5b**). No racemization of **5** at its chiral center was observed, as judged by both ^{1}H NMR and HPLC, in good agreement with previous reports for similar reactions.[10a] Compounds **5a** and **5b** were proven to be inseparable by TLC and flash chromatography and therefore used as a mixture for the subsequent attachment to the solid support. We chose 2-Cl-trityl resin because the phenolic alcohol on **5** can be loaded onto this solid support under mild conditions with high efficiency. In addition, the resin is compatible with Fmoc chemistry. A number of loading strategies were tested. It was found that using cesium carbonate together with pyridine as a mild base allowed different phenolic-*N*-Fmoc-α-amino-vinyl sulfones **5** to be loaded onto the resin with high efficiency (loading level = 0.43−0.52 mmol/g; Table 2).

Table 2. Yield of **5** and the Loading Efficiency on 2-Cl-Trityl Resin

5	yield (%)	**6** loading efficiency (mmol/g)
Fmoc-Leu	74	0.44
Fmoc-Lys(Boc)	78	0.43
Fmoc-Tyr(tBu)	72	0.52
Fmoc-Asp(tBu)	59	0.48
Fmoc-Phe	67	0.45

We next examined the utility of these vinyl sulfone-containing resins in the solid-phase synthesis of peptide vinyl sulfones. As an example, *N*-Fmoc-α-Leu-vinyl sulfone resin was used as the solid support, and a potential tetrapeptide inhibitor (e.g., H_2N-Gly-Tyr-Leu-Leu-vinyl sulfone) was synthesized using standard Fmoc-based peptide chemistry (see Scheme 2 for a representative synthetic strategy). Upon completion of the synthesis, the resulting product was cleaved

Scheme 2. Synthesis of the Activity-Based Probe[a]



[a] Reagents and conditions: (a) 20% piperidine in DMF, 30 min. (b) HBTU (4 equiv), HOBt (4 equiv), DIEA (8 equiv), Fmoc-AA-OH (4 equiv), preactivation for 3 min, then coupling for 2 h. (c) Cy3 (3 equiv), DIC (3 equiv), HOBt (3 equiv), DIEA (6 equiv), 12−18 h. (d) Reagent R.

from the resin and directly analyzed by reverse-phase HPLC and mass spectrometry to assess its purity: more than 90% of products generated were the desired products (Figure 1),

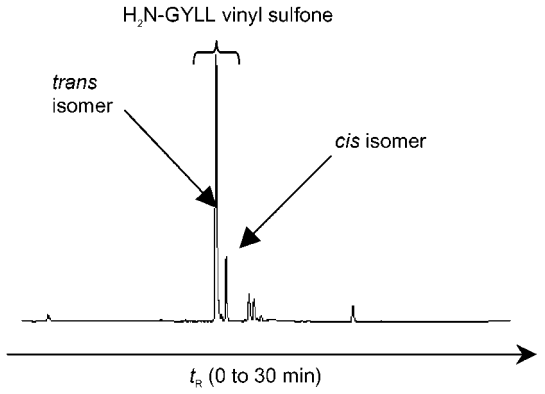


Figure 1. HPLC analysis of the crude peptide vinyl sulfone.

in which both the cis and trans isomers of the peptide vinyl sulfone were observed, in good agreement with previous reports.[4] In addition, no epimerized product was detected, further indicating that the Horner−Emmons condensation (e.g., **3** → **5** in Scheme 1) was indeed racemization-free.

We then extended the strategy to the solid-phase synthesis of a vinyl sulfone-containing, activity-based probe that targets

cysteine proteases. The design of the probe was as previously described.[6] Briefly, in addition to the vinyl-sulfone reactive unit, the probe also contains a tetrapeptide recognition unit, as well as a Cy3-containing fluorescence unit, which facilitates the detection of proteins upon labeling by the probe (Scheme 2). The probe **7** was synthesized using the *N*-Fmoc-α-Tyr(*t*Bu)-vinyl sulfone resin. The Cy3 dye was conveniently coupled onto the solid support at the end of peptide synthesis, using the standard DIC/HOBt/DIEA strategy. Upon cleavage of the resin, the resulting probe was precipitated with cold ether and purified to homogeneity by HPLC.

The probe was subsequently tested for selective labeling of cysteine proteases on the basis of their enzymatic activity (Figure 2). A panel of 12 enzymes was used, of which two were cysteine proteases. Only cysteine proteases (i.e., lanes 4 and 5) were selectively labeled, indicating the specific nature of the probe. To further confirm that the fluorescence labeling of the cysteine proteases by the probe is due to their enzymatic activity, the enzymes were first inactivated by heat and then treated with the probe followed by SDS-PAGE analysis. No labeling of the cysteine proteases was observed, indicating that the enzymatic activity is a prerequisite for the labeling reaction to occur.

In conclusion, we have successfully developed a facile and efficient approach that allows the solid-phase synthesis of peptide vinyl sulfones using standard Fmoc chemistry. This method circumvents numerous limits imposed by previously reported solid-phase methods.[4b,7] Peptide vinyl sulfones having different amino acids at the P_1 position may be readily synthesized in high yield and purity. The method may be used to synthesize vinyl sulfone-containing, activity-based probes to profile cysteine proteases in a proteome. Consequently, this strategy may provide a useful tool for the identification of new cysteine proteases and the generation of their potential inhibitors.

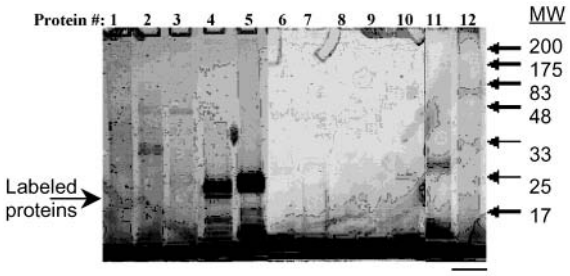


Figure 2. Activity-based protein profiling using probe **7**. Lane 1: Type I-S Alkaline Phosphatase, from bovine intestine. Lane 2: Type VIII Alkaline Phosphatase, from rabbit intestine. Lane 3: Type IV Alkaline Phosphatase, from porcine intestinal mucosa. Lane 4: Chymopapain, from papaya latex. Lane 5: Papain, from papaya latex. Lane 6: α-Chymotrypsin. Lane 7: β-Chymotrypsin. Lane 8: γ-Chymotrypsin. Lane 9: Proteinase K, from tritirachium album. Lane 10: Subtilisin, from bacillus licheniformis. Lane 11: Lysozyme, from chicken egg white. Lane 12: Lipase, from *Candida rugosa*. For details of the labeling reaction, see Supporting Information.

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Supporting Information Available: Experimental details and characterizations of compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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