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# Evaluation of Vinylsulfamides as Sulfhydryl Selective Alkylation Reagents in Protein Modification

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**Abstract**—The preparation of several model vinylsulfamides is described. Their excellent selective reactivity towards sulfhydryl group with regards to amino group has been demonstrated by the kinetics study between a model vinylsulfamide and cysteine or lysine at different pHs.


Michael acceptors such as vinylogous amino acid derivatives[1] and vinylsulfone-containing molecular entities[2,3] have been utilized in the design of novel protease inhibitors. In particular, the vinylsulfone moiety has recently been incorporated into several specific peptide sequences which resulted in potent inhibitors for proteasomes.[4,5] The latter have been implicated in a number of diseases; growing evidence has suggested that inhibitors of proteasomes may represent potentially new and unique treatments for inflammatory diseases[6] and cancer.[7] In another area, vinylsulfones were employed to functionalize polyethylene glycol (PEG)[8] and one of the potential applications for such functionalized PEG is to attach themselves to certain therapeutic proteins through alkylation. The PEGylated proteins are usually more stable and have longer in vivo half lives, which result in improved therapeutic profiles for these proteins. On the other hand, vinylsulfamides, which are structurally similar to vinylsulfones and may be easier to prepare, have not received attention in the aforementioned applications. In this report, we describe the synthesis of several model vinylsulfamides and the determination of their intrinsic reactivity that favors sulfhydryl over amino group.

The synthesis of all model sulfamides (Table 1) except compound **2c** is illustrated in Table 1, in which 2-chloroethanesulfonyl chloride was allowed to react with an equal molar amount of a particular amine in the presence of triethylamine (TEA). The role of TEA was not only to neutralize the first mole of HCl, resulting from the reaction between the sulfonyl chloride and the amine, but also to effect the elimination of the second mole of HCl, which led to the isolation of the desired vinylsulfamide in a single work up step. Because of this reason, two equivalents (or three equivalents in the case where the amine was used in its salt form) of TEA were required in the reaction. The initial attempt to prepare compound **2b** was made through the hydrolysis of **2a** in a mixture of 2 N NaOH/MeOH (3/10, v/v) at 45 °C for 3 h; nevertheless, the vinyl group was attacked by the hydroxyl anion to give compound **4** under this condition (Scheme 1).[9] The free acid, 4-(aminomethyl)benzoic acid, was therefore allowed to react directly with 2-chloroethanesulfonyl chloride (Scheme 2). The desired compound, **2b**, was formed along with a relative 40% of the corresponding dimer (**5**) as the by-product. The reaction mixture containing **2b** and **5** were then converted to their NHS esters and separated via column chromatography.[10] Based upon its anticipated different reactivity towards sulfhydryl (by the vinylsulfamide moiety) and amino (by the NHS ester moiety) groups, the purified compound (**2c**) appears to be a useful heterobifunctional cross-linking agent for proteins.[11] On the other hand, compound **3** could deliver the fluorescent label[12] to a specific site within proteins that contain a free cysteine residue. These expectations rely on the presumption that the vinylsulfamides really possess the intrinsic preferred reactivity towards sulfhydryl group, which has been proven experimentally and is described in the next paragraph.

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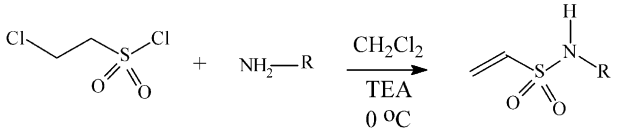
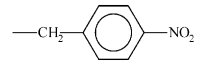
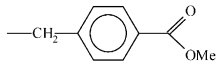
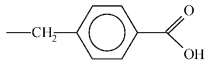
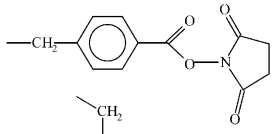
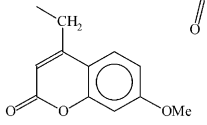
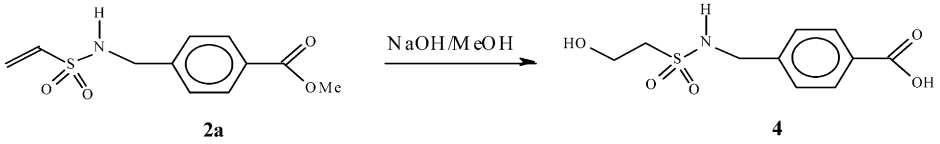
**Table 1.** Synthesis and structures of model vinylsulfamides[1]



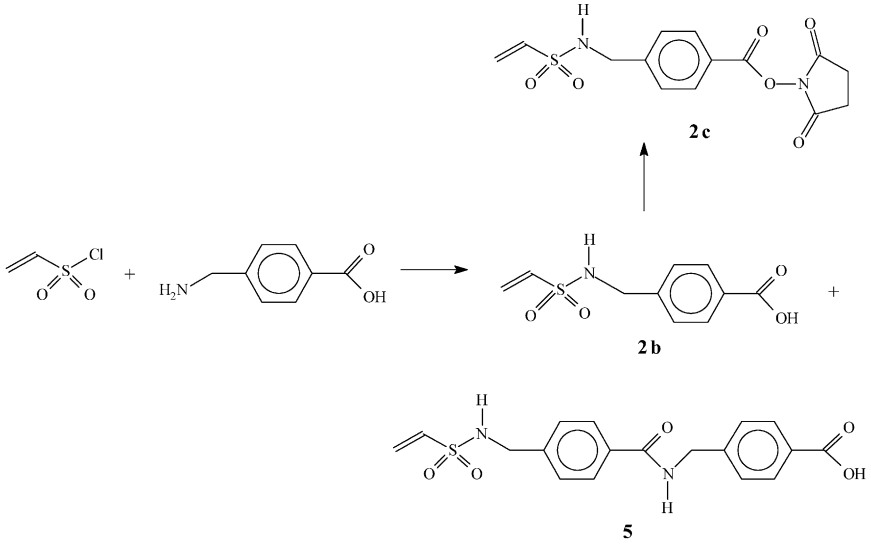
| Compd | R | Isolated Yield (%) |
|---|---|---|
| **1** |  | 47 |
| **2a** |  | 46 |
| **2b** |  | Note 2 |
| **2c** |  | 23 |
| **3** |  | 63 |

Notes: (1) General reaction procedure: to an ice–water cooled solution of 2-chloroethanesulfonyl chloride in methylene chloride (0.2 M) was added one equivalent of the amine (or its HCl salt) and one (or two) equivalent(s) of TEA, respectively. After the resulting mixture was stirred at 0 °C for 1 h, a second equivalent of TEA was added and the reaction was continued for another 1 h. The reaction mixture was then poured onto a mixture of saturated $NaHCO_3$/ice, followed by addition of more methylene chloride. The organic phase was separated and the aqueous phase extracted with methylene chloride (3×). All organic solutions were combined and washed with saturated $NaHCO_3$/ice–water (1/4, v/v, 1×), chilled 0.5 M HCl (2×), and brine (1×). The washed solution was then dried ($Na_2SO_4$) and evaporated to give the vinylsulfamide. (2) The compound was not isolated at this stage. It was directly converted into its NHS ester and then isolated through column chromatography.

It was demonstrated previously that the vinylsulfones showed good selectivity towards sulfhydryl group with regards to amino group.[8] Here, we evaluated the reactivity of the vinylsulfamides towards sulfhydryl and amino groups by measuring the half-lives of the model vinylsulfamide (**1**),[13] under different pHs at room temperature (21 ± 1 °C). The measurement experiments were performed in phosphate buffers where five times molar excess of cysteine or lysine was added versus **1** in order to achieve pseudo-first order reaction condition (experiment conditions listed in the Note under Table 2). The concentrations (A, as represented by peak Area) of the vinylsulfamide (**1**) was followed by HPLC and the Ln ($A/A_0$) was plotted against time (an example is shown in Fig. 1). The half-life was then calculated from the slope and the results are summarized in Table 2. Since the goal of the present study was to obtain qualitative kinetics, the reactions were performed in a thermostat-controlled laboratory rather than a preciously controlled temperature bath.[14] Nevertheless, the pseudo-first order plot displayed excellent linearity with a correlation coeffeciency ($R^2$) of 0.9967 (Fig. 1). At pH 8.1, vinylsulfamide **1** showed, respectively, a half-life of 27 min in the presence of five times more cysteine and a half-life of 7.4 days (or 177.4 h) in the presence of five times more lysine. In another word, the selectivity of the vinylsulfamide (**1**) is approximately 390 times in favor of sulfhydryl over amino group. Around neutrality (pH 7.2), **1** displayed a half-life of 77 min when cysteine was present; at the same pH; the half-life of **1** was too slow to measure in the presence of lysine within a limited time frame. On the other hand, the half-life of **1** decreased to 29.6 h at pH 9.2 when lysine was present, whereas in the case of cysteine at the same pH, the half-




**Scheme 1.**




**Scheme 2.**

**Table 2.** Summary of half-lives of vinylsulfamide **1** in the presence of five times molar excess of cysteine or lysine

| pH | 7.2 | 8.1 | 9.2 |
|---|---|---|---|
| Cysteine | 77 min | 27 min | ND1 |
| Lysine | ND2 | 7.4 days | 29.6 h |

Note: The measurement of the half-lives was performed in 50 mM phosphate buffers containing 10% EtOH and 5 mM EDTA at room temperature (21 ± 1 °C). The reaction solutions were monitored by an HP1100 HPLC system that was equipped with a Hypersil C18 column (4.6×150 mm, 5 μm) and a diode-array detector with detection set at 280 nm. The elution was effected by a gradient of 10–40% acetonitrile relative to the aqueous component consisting of 0.1% trifluoroacetic acid in water over a period of 30 min. Initial concentration of **1** was 1.33 mM. ND1: reaction rate was too quick to determine. ND2: reaction rate was too slow to measure within the time frame of the current study.

life was too quick to measure accurately with the methodology used in this study.

Morpurgo et al. measured the half-lives of several compounds bearing the sulfhydryl group in the presence of ten time molar excess of vinylsulfone PEG.[8] At pH 8.0, *N*-acetylcysteine had a half-life of 34 min, which is very comparable to the value of 27 min recorded in our case at pH 8.1. In their study, Morpurgo et al. was unable to determine the half-life of the amine-containing compound ($N^{\alpha}$-acetyllysine methyl ester) at pH 8.0, since no reaction could be detected after 24 h. This may be attributable to the amine assay method used in their study (TNBS assay[15]) which is less sensitive in detecting small changes than the HPLC method employed in the current study.

To test the alkylating ability of the vinylsulfamides towards the cysteine residue in a protein, compound **1** was treated with bovine serum albumin (BSA) which contains one free sulfhydryl group at Cys-34.[16] At a molar ratio of 20:1 (4 vs. BSA) at pH 7.0, 23% of the free sulfhydryl groups were found to be alkylated within 3 h at room temperature.[17] When the molar ratio was raised to 200:1, all of the sulfhydryl groups were reacted under the same condition.

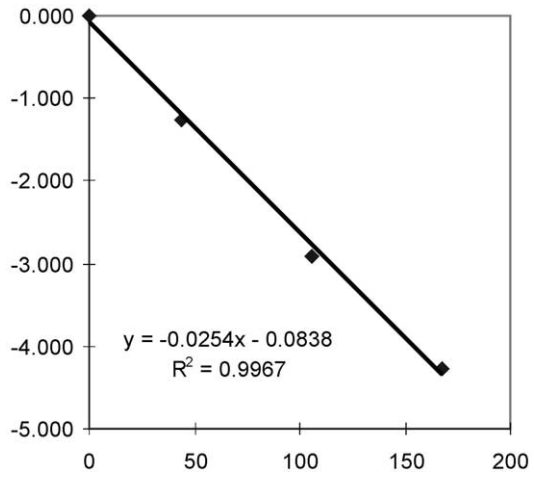



**Figure 1.** Linearity plot of the pseudo first order reaction between cysteine and vinylsulfamide **1** at pH 8.1. Note: The x-axis is the reaction time in min. The y-axis is Ln [$A/A_0$] where A is the concentration of vinylsulfamide **1** and $A_0$ is the concentration at time 0. The pseudo first order reaction constant, $k_1$, equals to the absolute value of the slope. The half-life, $t_{1/2}$, can therefore be calculated as $t_{1/2} = 0.693/k_1 = 27$ min.

In summary, we have shown that the vinylsulfamides are readily prepared in most cases and their excellent selectivity towards sulfhydryl group relative to amino group has been demonstrated by the kinetics study between the model vinylsulfamide and cysteine or lysine. Since amino-containing compounds are widely available or readily accessible, their conversion into the corresponding vinylsulfamides via reaction with 2-chloroethanesulfonyl chloride should bring about a variety of molecular entities that combine a particular functionality (as in the cases of compounds **2c** and **3**) with the sulfhydryl-selective vinylsulfamide moiety. Such resulting compounds will be useful for use as site-specific alkylation agents in protein/peptide modification and may also find their utility in the inhibitor design of proteasomes and certain proteases.

## References and Notes

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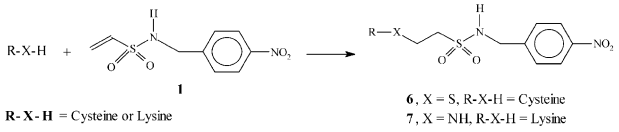
9. $^1$H NMR of the sodium salt of **4** (DMSO-$d_6$) δ 7.85 (d, 2H, $J = 8.1$ Hz), 7.70 (br, 1H), 7.25 (d, 2H, $J = 8.1$ Hz), 4.13 (s, 2H), 3.62 (t, 2H, $J = 6.4$ Hz), 3.21 (t, 2H, $J = 6.4$ Hz).
10. Procedures of the reactions and separation by column chromatography: to a solution of 2-choloroethanesulfonyl chloride (345 μL, 3.3 mmoL) in 31 mL of DMF that was cooled in an ice–water bath, was added 2-(aminomethyl)benzoic acid (500 mg, 3.3 mmoL) and TEA (878 μL, 6.3 mmoL), respectively. The resulting mixture was stirred at 0 °C for ~1 h and a second batch of TEA (502 μL, 3.6 mmoL) was added. The reaction mixture was allowed to warm to room temperature overnight and then filtered through a sintered glass funnel. The filtrate was evaporated in vacuo and the residue, after re-dissolved in a small amount of DMF, was poured into an ice-chilled 1 N HCl solution. The mixture was extracted with methylene chloride (5×) and the organic extract yielded 350 mg residue after removal of the solvent. $^1$H NMR analysis of the residue indicated that it contained **2b** and the dimer (**5**).[10a] The mixture was converted to their NHS esters according to the published procedure,[10b] and the reaction mixture was loaded onto a flash silica gel column which was eluted with ethyl acetate. The fast-eluted fractions gave the desired NHS ester, **2c**. $^1$H NMR ($CDCl_3$) δ 8.10 (d, 2H, $J = 8.3$

Hz), 7.47 (d, 2H, $J=8.3$ Hz), 6.49 (dd, 1H, $J_1=16.6$ Hz, $J_2=9.2$ Hz), 6.27 (d, 1H, $J=16.6$ Hz), 5.95 (d, 1H, $J=9.2$ Hz), 4.82 (t, 1H, $J=6.1$ Hz), 4.32 (d, 2H, $J=6.1$ Hz), 2.92 (s, 4H). (a) Part of the 2-chloroethanesulfonyl chloride molecules apparently acted as the activation reagent by reacting with **2b** to form a 'mixed anhydride' intermediate. Attack of this intermediate by a second molecule of **2b** yielded the dimer **5**. (b). Li, M.; Tsai, S.-F.; Rosen, S. M.; Wu, R. S.; Reddy, K. B.; DiCesare, J.; Salamone, S. J.. *J. Agric. Food Chem.* **2001**, *49*, 1287.

11. (a) For a comprehensive review of heterobifunctional cross-linking reagents, see: (a)Wong, S. S. *Chemistry of Protein Conjugation and Cross-Linking*; CRC: Boca Raton, FL, 1991. (b) Hermanson, G. T. *Bioconjugate Techniques*; Academic Press: San Diego, CA, 1996.

12. Characterization of **3**: $^1$H NMR (400 MHz, DMSO-$d_6$) δ 8.05 (t, 1H, $J=5.9$ Hz), 7.75 (d, 1H, $J=8.8$ Hz), 7.03 (d, 1H, $J=2.5$ Hz), 6.98 (dd, 1H, $J_1=8.8$ Hz, $J_2=2.5$ Hz), 6.86 (dd, 1H, $J_1=16.4$ Hz, $J_2=9.9$ Hz), 6.31 (s, 1H), 6.12 (d, 1H, $J=16.4$ Hz), 6.04 (d, $J=9.9$ Hz), 4.31 (d, 2H, $J=5.9$ Hz), 3.87 (s, 3H). $^{13}$C NMR (100 MHz, proton-decoupled, DMSO-$d_6$) δ 162.45, 160.11, 154.89, 152.39, 136.65, 126.03, 125.78, 112.21, 110.84, 109.76, 100.90, 55.94, 42.33. FAB-MS: $m/z$ 296.2 ($M+H^+$). IR: 3240, 3094, 1720, 1610, 1139 cm$^{-1}$. UV (MeOH) 217 (ε 19,117), 322 nm (ε 13,773). The corresponding fluorescent 4-(aminomethyl)-7-methoxycoumarin was synthesized according to the procedure of Li and White: Li, M.; White, E. H. *Bioconjugate Chem.* **1994**, *5*, 454.

13. (1) Characterization of **1**: $^1$H NMR (200 MHz except where specified) ($CDCl_3$) δ 8.18 (d, 2H, $J=8.8$ Hz), 7.51 (d, 2H, $J=8.8$ Hz), 6.51 (dd, 1H, $J_1=9.6$ Hz, $J_2=16.4$ Hz), 6.25 (d, 1H, $J=16.4$ Hz), 5.96 (d, 1H, $J=9.6$ Hz), 5.11 (t, 1H, $J=6.4$ Hz), 4.30 (d, 2H, $J=6.4$ Hz). ESI-MS: $m/z$ 243 ($M+H^+$). (2) The adduct (**6**) formed between cysteine and **1** was isolated and characterized by $^1$H NMR: ($D_2O$/DCl) δ 7.41 (d, 2H, $J=8.7$ Hz), 6.79 (d, 2H, $J=8.7$ Hz), 3.62 (s, 2H), 3.56 (m, 1H), 2.65 (m, 2H), 2.49–2.27 (m, 2H), 2.15 (m, 2H). (**3**) The yield for the adduct (**7**) derived from lysine and **1** was quite low isolation of the product was not attempted.




14. Such a practice has been adopted in the kinetics study of Morpurgo et al.[8]

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