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Kinetics and Mechanism of the Addition of Nucleophiles to α,β-Unsaturated Thiol Esters

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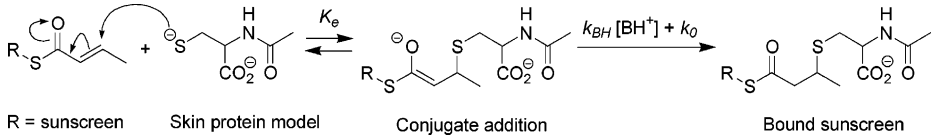

Compounds containing the UV-absorbing chromophores *p*-methoxycinnamate, *p*-methoxycinnamide, or anthranilate and an α,β- or α,β,γ,δ-unsaturated thiol ester (crotonyl or sorboyl) have been prepared. These compounds are subject to nucleophilic attack at the C=C conjugated to the thiol ester carbonyl group. The kinetics of the reactions of these thiol esters with *N*-acetyl-L-cysteine (NAC), *N*-acetylcysteamine, and N^2-acetyl-L-lysine (NAL) have been studied, and the thiol addition products have been identified. The reaction rates increased at higher pH, and the reaction of NAC thiolate with a crotonyl thiol ester in 1:1 (v/v) acetonitrile/aqueous HEPES exhibited buffer catalysis as a result of protonation of the enolate intermediate. At the same concentration, NAC underwent ~300-fold more reaction than NAL with a crotonyl thiol ester at pH 9.8. Additionally, a crotonyl thiol ester was found to be 7.9 times more reactive than a sorboyl thiol ester toward NAC addition. These unsaturated thiol esters may serve as a means of covalently binding UVA and UVB sunscreens to the outer layer of skin to provide long-lasting protection.

Introduction

One of the more serious daily assaults on skin is exposure to solar ultraviolet (UV) radiation. Such exposure can have a variety of detrimental effects,[1] including induction of skin cancer.[2] Shorter wavelength radiation (UVB, 290−320 nm) is generally responsible for formation of cyclobutane pyrimidine dimers and other photoproducts, and it causes specific *p53* tumor suppressor gene mutations, which are thought to be an early, essential event in skin cancer.[3,4] The more abundant UVA radiation (320−400 nm) can penetrate more deeply into the skin than UVB, reaching the dermis,[5] and it has been linked to skin cancer, both nonmelanoma and malignant melanoma.[6] Effects of chronic exposure to UVA include photoaging of the skin, characterized by increased pigmentation and stratum corneum thickness, decreased hydration, and loss of elasticity.[7] Additionally, UV exposure results in immunosuppression.[8]

Numerous studies have demonstrated the effectiveness of sunscreen use in preventing pyrimidine dimer formation,[9] p53 mutations,[10] precancerous lesions, such as actinic keratoses,[11,12] skin cancer,[13] photoaging of skin,[14,15] and also photoimmuno-

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suppression.[4,14] In addition to dramatic tumor prevention by sunscreens applied to mice prior to UVB exposure (100% vs 2% tumor development) and significant reduction in p53 mutations,[16] sunscreen use in a human clinical study[15] showed that daily use of a broad-spectrum-absorbing sunscreen protected against many of the changes associated with photoaging. An Australian study comparing daily sunscreen use to discretionary use only (control group) over a period of 4.5 years found a significant decrease in the incidence of squamous but not basal cell carcinomas.[17]

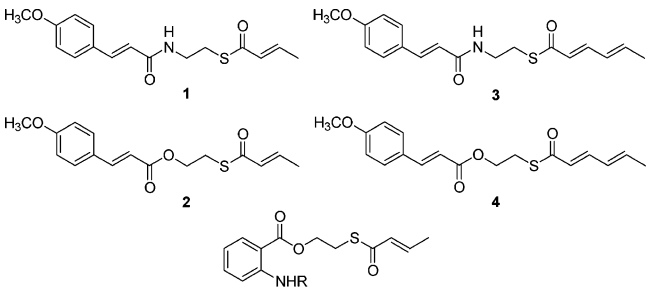
Users typically fail to apply and maintain the 2 mg/cm^2 coverage required to achieve the specified SPF.[18] A sunscreen capable of covalently bonding to skin would potentially avert loss, for example, due to rubbing off and exposure to water. Such a strategy would entail linking a UV-absorbing moiety to a skin-bonding functionality that would react with nucleophilic groups (e.g., $-NH_2$, $-SH$) in skin proteins. To avoid generation of the byproducts inherently produced in nucleophilic substitution reactions, formation of adducts of nucleophiles via conjugate addition to α,β-unsaturated electrophiles seemed preferable. Although numerous unsaturated systems are known to undergo conjugate addition (e.g., α,β-unsaturated ketones, *N*-alkylmaleimides, α,β-unsaturated nitriles), most appear too reactive for topical, cutaneous use.

Thiol esters are electrophilic and have utility in the native chemical ligation of proteins via intramolecular rearrangement. Incorporation of unsaturation in conjugation with the thiol ester carbonyl group offers a C═C for conjugate addition by nucleophiles.[19−21] The work described herein explores the reactivity of the C═C in α,β-unsaturated thiol esters. The kinetics and mechanism of conjugate addition reactions of nucleophiles have been studied, and a comparison to other electrophiles that undergo conjugate addition reactions has been made to assess the suitability of α,β-unsaturated thiol esters as mildly reactive bonding agents for sunscreens to nucleophilic groups in skin.

Results and Discussion

In this study, *p*-methoxycinnamoyl and anthranilate moieties have been modified to contain a pendant α,β- or α,β,γ,δ-unsaturated thiol ester, as shown below (**1**−**6**). These UV-absorbing compounds are designed for covalent attachment to nucleophilic groups (e.g., $-NH_2$, $-SH$) in skin proteins in the uppermost layers of the epidermis (e.g., the stratum corneum). The thiol ester functional group provides increased reactivity of the C═C toward conjugate addition by nucleophiles, compared to an ordinary oxygen ester,[22] and it is 20−30 times more stable toward acid hydrolysis, although it is of comparable stability to oxygen esters toward alkaline hydrolysis.[23] Nucleophiles, such as DMAP[24,25] and Ph_3P,[25] readily add to the C═C in conjugation with the C(═O)S functionality. Thiols also add to α,β-unsaturated systems containing electron-withdrawing groups, such as vinyl[26] sulfones, sulfonates, and sulfonamides, enones,[27] and α,β-unsaturated esters[28] and imides.[29] α,β-Unsaturated thiol esters undergo conjugate addition reactions with thiols,[22,30] but kinetic data are lacking.

The UVB-absorbing *p*-methoxycinnamoyl chromophore is widely used in sunscreen formulations as the ethylhexyl ester (FDA-allowed concentrations: 2−7.5%), and the UVA-absorbing anthranilate chromophore is generally found as the menthyl ester (FDA-allowed concentrations: 3.5−5%). For the purpose of skin bonding and the mechanistic studies reported herein, the alkyl groups normally attached to these UV-absorbing moieties were replaced by unsaturated thiol ester functionalities, as shown below:



5 R=CH_3
6 R=$COCHCHCH_3$

It is possible that differing solubility characteristics of the amides **1** and **3** compared to those of the esters **2**, **4**, **5**, and **6** will influence skin permeability and may thereby alter the site of covalent attachment of the compound within the stratum corneum.

Synthesis of the *p*-methoxycinnamoyl-containing UV absorbers is outlined in Scheme 1. The acid chloride of *p*-methoxycinnamic acid **7** was treated with cystamine to give disulfide **8** or with hydroxyethyl disulfide to give disulfide **10**. Reduction to the corresponding thiols **9** and **11** was accomplished by treatment with zinc/HCl. Subsequent reaction with crotonyl chloride or sorboyl chloride gave compounds **1** and **2** or **3** and **4**, respectively.

Preparation of the anthranilate-containing UV absorbers is outlined in Scheme 2. Reaction of isatoic anhydride or *N*-methylisatoic anhydride in DMF with hydroxyethyl disulfide in the presence of 10% DMAP gave the disulfides **12** or **13**. These disulfides were each reduced by zinc and acid to the corresponding thiols **14** and **15**. In the case of the unmethylated anthranilate, coupling with crotonyl chloride/pyridine produced the dicrotonyl compound **6**. The UV spectrum of this compound was shifted somewhat toward the UVB region (λ_{max} = 315,

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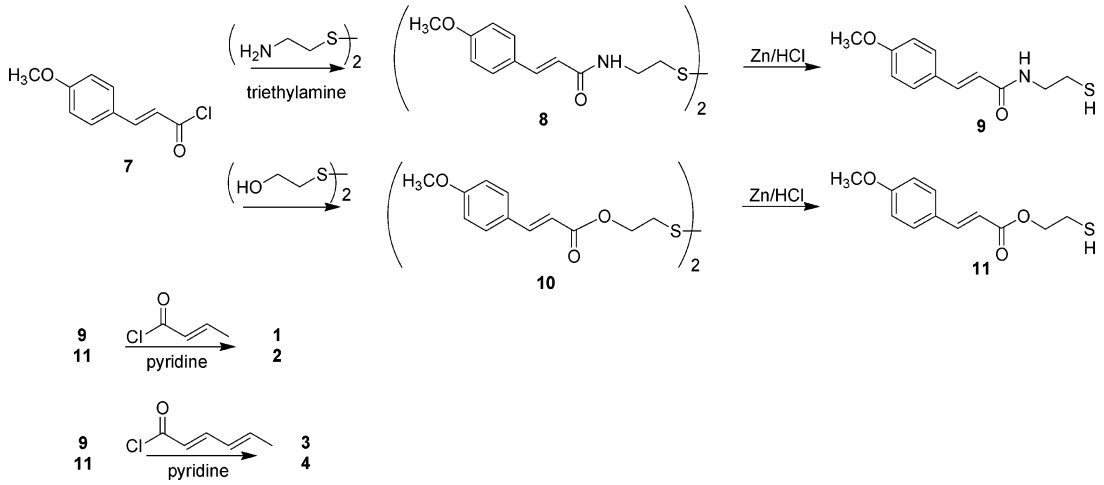
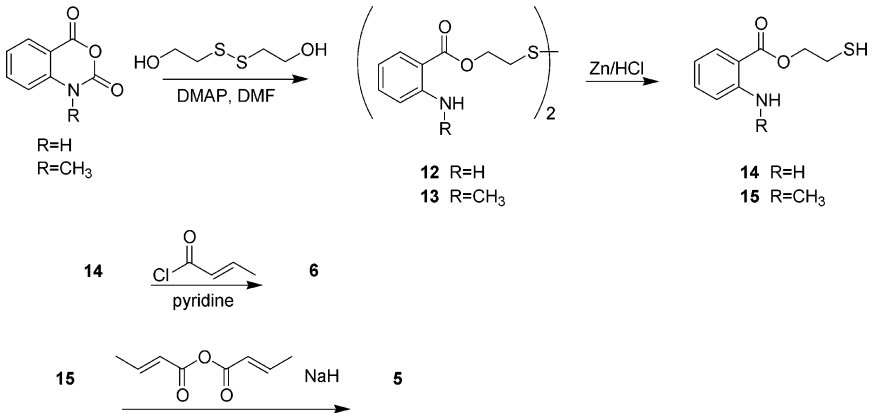
SCHEME 1



SCHEME 2



264 nm, in 95% ethanol). The *N*-methylanthranilate derivative **13**, however, upon treatment with crotonic anhydride/NaH, produced crotonate thiol ester **5**, which had a UV absorption spectrum characteristic of unsubstituted anthranilates (λ_{max} = 357, 255 nm, 95% ethanol), and it thus serves as a UVA absorber.

It was found that conjugate addition of nucleophilic groups to the α,β-unsaturated thiol ester readily occurred, giving a covalent adduct. The reactions of compound **1** or **2** with *N*-acetylcysteamine, *N*-acetyl-L-cysteine, or N^2-acetyl-L-lysine, as protein model compounds, to form adducts **16**, **17**, and **18** are shown in Figure 1.

Thiol esters are more reactive toward conjugate addition than are ordinary oxygen esters,[31] which can be attributed to the reduced resonance stabilization in thiol esters.[32,33] This is at least in part a consequence of the poorer 2p−3p orbital overlap in the former, although it is not insignificant.[34]

An NMR experiment was carried out to demonstrate the reactivity of the α,β-unsaturated thiol ester functional group and to identify the structure of the product. The spectrum was recorded before (Figure 2, top) and after (Figure 2, middle) the addition of 1 equiv of *N*-acetylcysteamine to **2**. No reaction is evident at this time. Within 2 min of introduction of 0.1 equiv of a base (1,5-diazabicyclo[4.3.0]non-5-ene, DBN), however, addition of the cysteamine thiol to the crotonate double bond had occurred. This is clearly evidenced by the disappearance of the signal due to the vinyl protons (doublet at δ 6.15 ppm, multiplet at δ 6.96 ppm) and the shift of the signal due to the methyl group protons from δ 1.9 to 1.3 ppm (Figure 2, bottom). Additionally, the triplet at δ 1.3 ppm due to the cysteamine SH proton is no longer present after reaction with **2** has occurred. COSY, HMBC, and HMQC spectra confirmed the structure of the product, **16**, and similar results were found with **1** (Supporting Information).

An NMR study of the reaction of sorboyl thiol ester **4** with *N*-acetylcysteamine revealed that it reacted more slowly than the analogous crotonate **2**. Fifteen minutes after addition of 0.1 equiv of DBN, 64% of the sorboyl vinyl protons remained, while 36% appeared in a mixture of monoadducts, as evidenced by the appearance of two multiplets at δ 5.25 and 5.5 ppm in the NMR spectrum. The kinetics of a competitive reaction of *N*-acetylcysteamine with **2** and **4** were followed by HPLC, and the results are described below. Reaction of the sorboyl thiol ester **3** with *N*-acetylcysteamine was also slower than that of the corresponding crotonyl thiol ester **1**. Five minutes after addition of 0.2 equiv of DBN, 45% of the sorboyl vinyl protons remained, while 55% appeared in a mixture of monoadducts.

Conjugate addition to form a covalent adduct also occurred in the case of the anthranilate-containing thiol esters. An NMR

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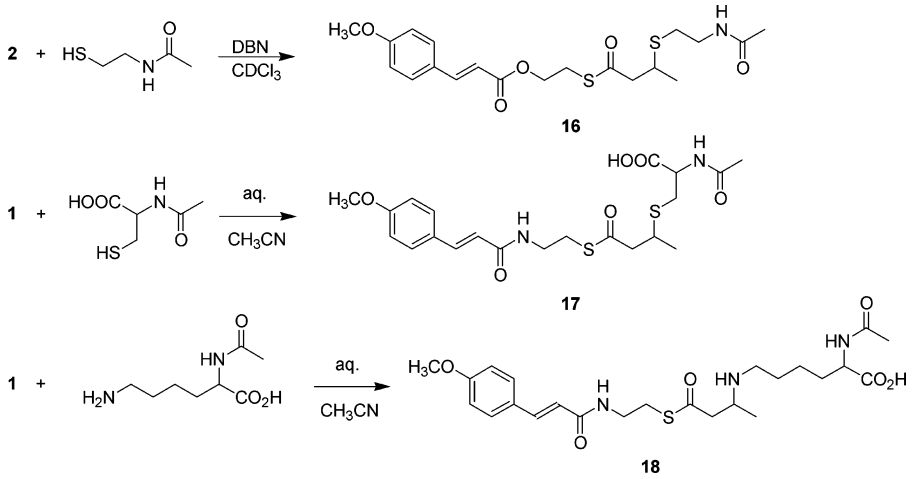


FIGURE 1. Conjugate addition of *N*-acetylcysteamine, *N*-acetyl-L-cysteine, or N^2-acetyl-L-lysine to **1** or **2** to form the adducts **16**, **17**, and **18**.

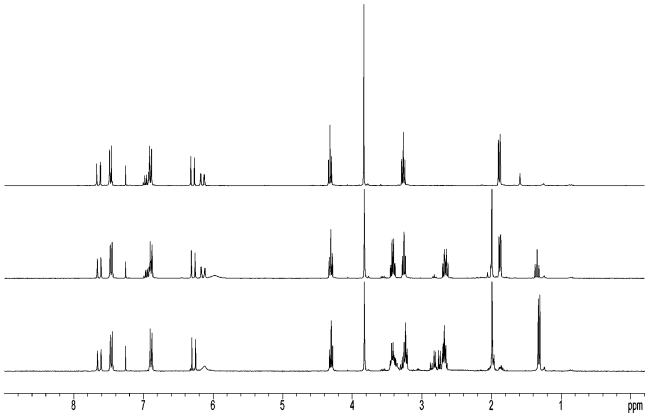


FIGURE 2. Reaction of **2** with *N*-acetylcysteamine and DBN. Top spectrum: **2** in $CDCl_3$. Middle spectrum: **2** with 1 equiv *N*-acetylcysteamine. Bottom spectrum: 2 min after addition of 0.1 equiv of DBN.

study of the addition of *N*-acetylcysteamine to **5** found that, 2 min after addition of 0.1 equiv of DBN, 50% of the thiol ester had reacted to form an adduct analogous to that shown in Figure 1 (Supporting Information). With compound **6**, 75% of the thiol ester had reacted to form an adduct after 2 min. Only the *S*-crotonyl group is reactive in this molecule; the *N*-crotonyl group remained unchanged in the presence of DBN/cysteamine (Supporting Information).

Kinetics of the addition of amino acid derivatives to the thiol esters was followed by HPLC. Reactions were carried out in a 1:1 mixture (v/v) of acetonitrile and 72 mM HEPES buffer at 23 °C. All points are the average of at least two determinations. Conjugate addition of *N*-acetyl-L-cysteine (NAC) to **1** was carried out under pseudo-first-order conditions, with greater than 10-fold excess *N*-acetyl-L-cysteine over **1**. The observed rate equation is

$$v = k_{\text{obs}}\,[\mathbf{1}] \tag{1}$$

Data were plotted according to the pseudo-first-order rate equation

$$\ln(A_t/A_o) = -k_{\text{obs}}\,t \tag{2}$$

A plot of the data from a typical reaction is shown in Figure 3.

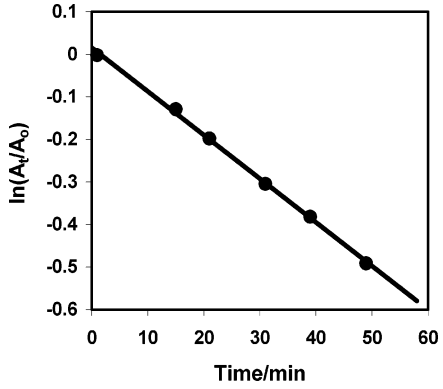


FIGURE 3. Kinetics of the reaction of 20.0 mM *N*-acetyl-L-cysteine and 0.77 mM **1** in 1:1 (v/v) acetonitrile/72 mM HEPES (aqueous buffer at pH 7.95) are shown. Reaction progress was monitored by the disappearance of reactant **1** by HPLC.

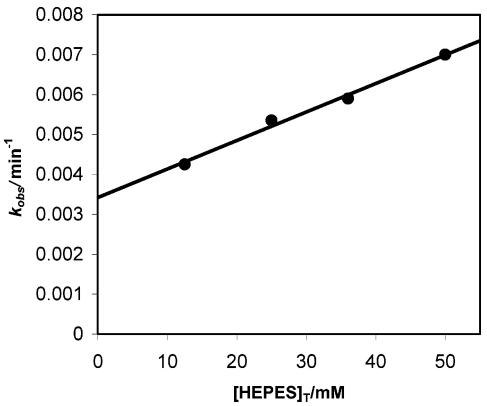
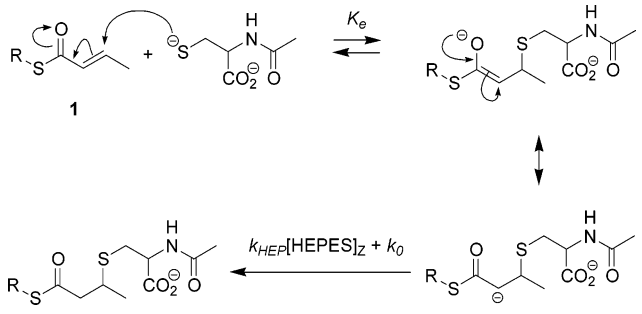


FIGURE 4. Reaction of **1** with *N*-acetyl-L-cysteine (25 mM) in HEPES-buffered 1:1 (v/v) water/acetonitrile, final pH 7.79. The line is the least-squares fit to the data ($r^2 = 0.99$). $[\text{HEPES}]_T$ is neutral HEPES zwitterion concentration plus HEPES anion concentration.

A study of the effect of varying the concentration of HEPES buffer revealed buffer catalysis, as shown in Figure 4.

The observation of buffer catalysis suggests the mechanism shown in Scheme 3, in which rapid attack of NAC thiolate is followed by rate-limiting protonation of the intermediate enolate ion by acids, such as neutral zwitterionic HEPES in solution. Equilibrium favors protonation of the enolate, as enolates of simple thiol esters are only weakly acidic ($pK_a \sim 21$).[34]

SCHEME 3



The mechanism in Scheme 3 exhibits general base catalysis, with the following rate law, in which $[HEPES]_z$ is neutral HEPES zwitterion concentration and K_e is defined in Scheme 3:

$$\frac{v}{[\mathbf{1}]} = k_{obs} = K_e\,[NAC\text{-}S^-](k_{HEP}[HEPES]_z + k_0) \quad (3a)$$

In terms of the total HEPES concentration (i.e., zwitterion and anion), $[HEPES]_T$, and the fraction of $[HEPES]_T$ present in zwitterionic form, f_z, eq 3a becomes

$$\frac{v}{[\mathbf{1}]} = k_{obs} = K_e\,f_{thiolate}\,[NAC]_T\,(k_{HEP}\,f_z\,[HEPES]_T + k_0) \quad (3b)$$

Rate-limiting protonation of anionic addition intermediates has been observed in the addition of thiolates to α,β-unsaturated nitriles[35] and to cyanamide.[36] In the case of *N*-ethylmaleimide, at least partial rate-limiting protonation of the intermediate imide anion was detectable but was relatively unimportant.[37] Carbon−sulfur bond formation, however, is rate limiting in the base-catalyzed addition of thiols to α,β-unsaturated ketones.[38]

The neutral form of HEPES (i.e., the zwitterionic form whose nitrogen atom is protonated) would be required for proton transfer to the enolate shown in Scheme 3. Given the pK_a of HEPES of 7.80 in 1:1 (v/v) H_2O/acetonitrile and that of NAC of 10.77, the fraction of HEPES present in zwitterionic form, f_z, is 0.51 and the fraction of total NAC in thiolate form, $f_{thiolate}$, is 1.05×10^{-3} at pH 7.79. The slope of the line in Figure 4, $(7.14 \pm 0.47) \times 10^{-2}$ M^{-1} min^{-1}, equals the slope of eq 3b with HEPES as the variable ($K_e\,f_{thiolate}\,[NAC]_T\,k_{HEP}\,f_z$), which gives $K_e k_{HEP} = (5.4 \pm 0.4) \times 10^3$ M^{-2} min^{-1}. The *y*-intercept, $(3.42 \pm 0.16) \times 10^{-3}$ min^{-1} (Figure 4), equals $K_e\,f_{thiolate}$-$[NAC]_T\,k_0$, which gives $K_e k_0 = (1.3 \pm 0.1) \times 10^2$ M^{-1} min^{-1}. The ratio then gives $k_{HEP}/k_0 = 41 \pm 3$ M^{-1}, which allows calculation of the ratio of buffer-catalyzed product formation to spontaneous protonation (e.g., by water) at different values of HEPES zwitterion concentration as [HEPES zwitterion] × k_{HEP}/k_0 (e.g., equal contributions at 24 mM HEPES zwitterion). Thus, protonation of the enolate intermediate by proteins in the skin could have an accelerating effect on the skin-binding reaction of sunscreen molecules, such as **1**.

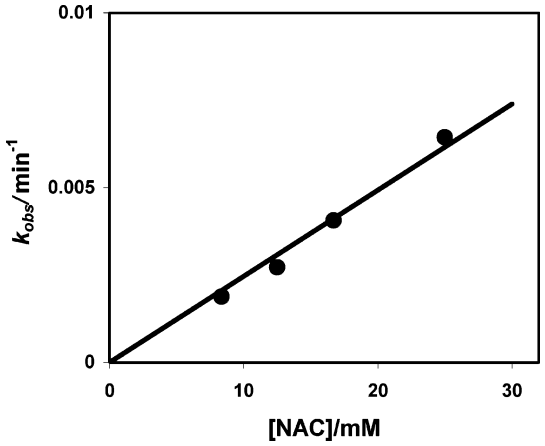


FIGURE 5. Reaction of **1** with varying concentrations of *N*-acetyl-L-cysteine in 1:1 (v/v) water/acetonitrile containing 36 mM HEPES at a final pH of 7.79. The line is a least-squares fit of eq 3 to the data ($r^2 = 0.98$).

The effect of varying the concentration of *N*-acetyl-L-cysteine was also studied, and the data are shown in Figure 5. The reaction rate was dependent on *N*-acetyl-L-cysteine concentration as expected.

The slope of the line in Figure 5 equals the slope of eq 3b with [NAC] as the variable, which gives $K_e\,f_{thiolate}(k_{HEP}\,f_z$-$[HEPES]_T + k_0) = 0.247$ M^{-1} min^{-1}. Calculation of this quantity by use of the values determined from Figure 4 was in good agreement, giving 0.240 M^{-1} min^{-1}.

Nucleophilic attack by the thiolate anion of *N*-acetyl-L-cysteine on **1** would be expected to be much faster than attack by the neutral *N*-acetyl-L-cysteine sulfhydryl group, as the neutral thiol is much less nucleophilic than the thiolate in conjugate addition reactions.[28,29,39,40] Conjugate addition of thiolates to Michael acceptors has been studied theoretically, as well.[41] A pH−rate profile for the reaction of **1** and *N*-acetyl-L-cysteine is shown in Figure 6. Indeed, the reaction was accelerated with increasing pH of the solution.

To assess the relative reactivities of the α,β-unsaturated and the α,β,γ,δ-unsaturated systems, a competitive experiment with crotonyl and sorboyl thiol esters was carried out. A 1:1 mixture of **1** and **3** was treated with *N*-acetyl-L-cysteine in 36 mM HEPES in 1:1 acetonitrile/water, pH 8.23. The results, presented in Figure 7, show that the crotonate is 7.9-fold more reactive than the sorbate.

A comparison of the reactivity of the thiol esters studied herein with the reactivity of the widely studied *N*-ethylmaleimide (NEM) can be made. The reactions of alkyl and aryl thiolates in water and 95% ethanol, respectively, with NEM have second-order rate constants[29,37] in the range of $\sim 1-20 \times 10^6$ M^{-1} min^{-1}. As shown above, thiolate addition to thiol ester **1** occurs with a second-order rate constant (in the absence of buffer) equal to $k_{obs}/(f_{thiolate}[NAC]_T) = K_e k_0 \sim 1.3 \times 10^2$ M^{-1} min^{-1}, considerably slower than NEM additions, although more sensitive to buffer catalysis.

The 6-amino group of lysine in skin proteins would also be expected to serve as a nucleophile capable of adding to **1**, so

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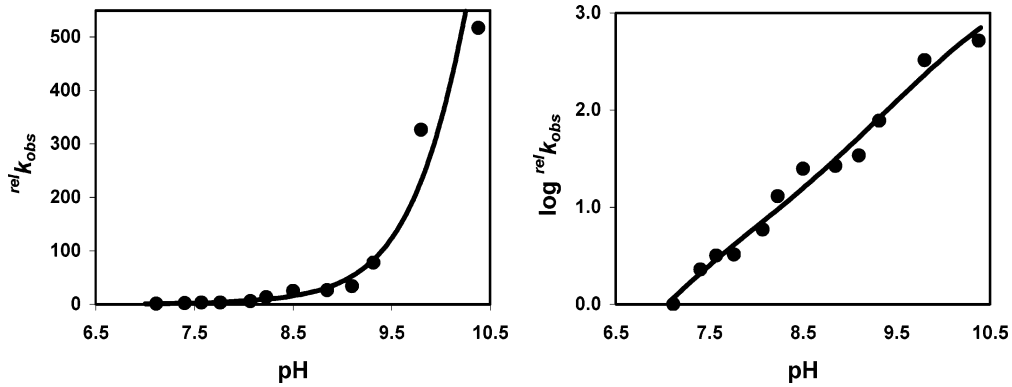

FIGURE 6. The pH−rate profile for the reaction of **1** with *N*-acetyl-L-cysteine. Left panel: relative k_{obs} ($^{rel}k_{obs}$) versus pH ($k_{obs} \equiv 1.0$ at pH 7.12). Right panel: log ($^{rel}k_{obs}$) versus pH. The reactions were carried out in 1:1 water/acetonitrile containing 36 mM HEPES at the final pH specified. The lines are the result of a nonlinear least-squares fit (see Experimental Methods) of eq 3b, with k_{HEP}/k_0 set equal to 41 M^{-1} (as determined from Figures 4 and 5).

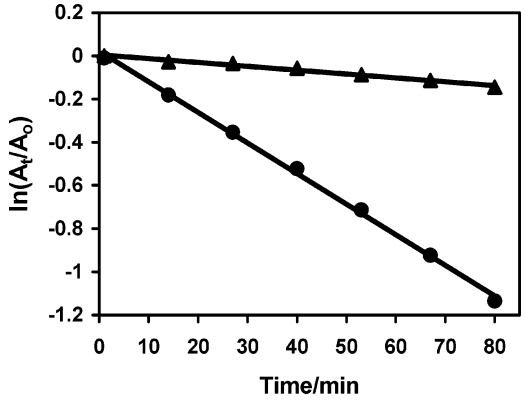

FIGURE 7. Comparison of the relative reactivities of the crotonyl thiol ester **1** (circles) and the sorboyl thiol ester **3** (triangles). A solution containing 0.77 mM **1** and 0.77 mM **3** in acetonitrile was mixed with an equal volume of 50 mM *N*-acetyl-L-cysteine in 72 mM HEPES (aqueous buffer at pH 7.95), and the disappearance of the thiol esters was monitored by HPLC.

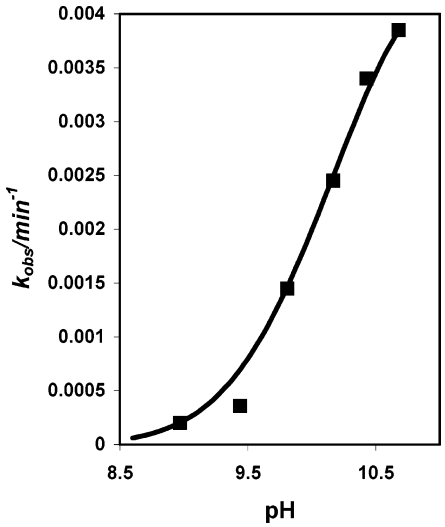

FIGURE 8. The pH−rate profile for the reaction of **1** (0.77 mM) with N^2-acetyl-L-lysine (14.3 mM). The reactions were carried out at the final pH specified in 1:1 (v/v) water/acetonitrile containing 76 mM sodium borate buffer.

model studies of the reaction of N^2-acetyl-L-lysine (NAL) with **1** were carried out. A pH−rate profile for the reaction is shown in Figure 8.

A comparison of relative reactivities of NAC and NAL can be made. For example, at pH 7.8, k_{obs} for NAC at 25 mM is 0.00342 min^{-1} (*y*-intercept of plot in Figure 4). Correction to 14.3 mM NAC (×14.3/25) and for the relative rate increase for pH 9.8 (×231, from Figure 6) allows comparison to k_{obs} for 14.3 mM NAL at pH 9.8 (Figure 8, if there is no significant buffer catalysis by boric acid). The resulting factor of ∼300 times greater reaction of NAC reveals that thiols are considerably more reactive in mildly alkaline solution, but the relative abundances and availability for reaction of cysteinyl and lysyl groups in skin will determine the actual major sites of reaction of compounds like **1** with skin.

Experimental Methods

A. Syntheses of *p*-Methoxycinnamates and *p*-Methoxycinnamides. A.1. *p*-Methoxycinnamoyl chloride (7). To 15.02 g of 4-methoxycinnamic acid in 200 mL of dry benzene with one drop of pyridine was added a 2.4-fold excess of thionyl chloride. Care was taken to trap the HCl vapor formed. The mixture was refluxed overnight, after which the solvent and excess thionyl chloride were removed by rotary evaporation. Repeated addition of benzene and evaporation removed the last traces of thionyl chloride to give a dark yellow solid (16.14 g, 97%). Mp: 67−69 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 3.87 (s, OC*H*$_3$), 6.51 (d, J = 15.0 Hz, C*H*CO), 6.94 (dd, J = 6.9 Hz, 1.8 Hz, aromatic C(3)*H*, C(5)*H*), 7.53 (dd, J = 6.9 Hz, 1.8 Hz, aromatic C(2)*H*, C(6)*H*), 7.79 (d, J = 15.3 Hz, COCHC*H*). ^{13}C (75.46 MHz, $CDCl_3$): δ (ppm) 55.5, 62.1, 114.7, 119.4, 125.7, 131.1, 150.6, 162.9, 166.0.

A.2. Bis(*p*-methoxycinnamate) ester of 2-hydroxyethyl disulfide (10). Preparation was according to the literature.[42] To 60 mL of benzene were added 4.25 g (0.0216 mol) of **7** and 1.66 mL (0.0135 mol) of 2-hydroxyethyl disulfide. The mixture was refluxed for 1.5 h and allowed to stir at room temperature overnight. Solvent was removed by rotary evaporation, and the residue was taken up in chloroform, and the organic phase was washed with water. The aqueous layer was back-extracted with chloroform. The combined organic layers were dried over anhydrous $MgSO_4$ and evaporated to give 5 g of crude solid. This material was recrystallized from absolute ethanol to give 3.6 g (70%) of white solid. Mp: 80−81 °C (found), 81.5 °C (lit).[42] ^{1}H NMR ($CDCl_3$): δ (ppm) 3.03 (t, J = 6.6 Hz, CH_2S), 3.83 (s, OCH_3), 4.47 (t, J = 6.8 Hz, CH_2N), 6.30 (d, J = 15.9 Hz, C*H*CO), 6.89 (d, J = 9.0 Hz, aromatic C(3)-

(42) Jung, L.; Richard, A.; Navarro, R. European Patent No. FR2504530, 1982.

H, C(5)*H*), 7.46 (d, $J = 8.7$ Hz, aromatic C(2)*H*, C(6)*H*), 7.65 (d, $J = 15.9$ Hz, C*H*CHCO). ^{13}C (75.46 MHz, $CDCl_3$): δ (ppm) 37.4, 55.2, 62.1, 114.2, 114.8, 126.9, 129.7, 144.9, 161.3, 166.8.

A.3. 2-Mercaptoethyl *p*-methoxycinnamate (11). To 100 mL of ethanol/acetonitrile, 1:1 by volume, was added 1.2 g of **10**. This was treated with 3.5 g of zinc dust and 1 mL of concentrated HCl, and the reaction mixture was stirred for 2 h at room temperature. Complete reduction to the thiol was verified by TLC (silica, 20% acetone in hexanes; disulfide $R_f = 0.24$, thiol $R_f = 0.45$). The reaction mixture was filtered to remove zinc and zinc salts, and the solvents were removed by rotary evaporation. The residue was dissolved in chloroform, the resulting solution was washed once each with water and dilute aqueous sodium bicarbonate, and the organic layer was dried over anhydrous $MgSO_4$. Removal of the solvent by rotary evaporation gave **11** as an oil that solidified in the cold to a white waxy material. ^{1}H NMR ($CDCl_3$): δ (ppm) 1.54 (t, $J = 8.4$ Hz, S*H*), 2.82 (dt, $J = 6.6$, 9 Hz, CH_2SH), 3.84 (s, OC*H*), 4.32, (t, $J = 6.6$ Hz, CH_2O), 6.31 (d, $J = 15.9$ Hz, CHCO), 6.9 (d, $J = 6.3$ Hz, aromatic C(3)*H*, C(5)*H*), 7.48 (d, $J = 6.9$ Hz, aromatic C(2)*H*, C(6)*H*), 7.66 (d, $J = 15.9$ Hz, C*H*CHCO).

A.4. *S*-Crotonyl-2-mercaptoethyl 4-methoxycinnamate (2). Thiol **11** (0.6 g, 2.5 mmol) and 1.6 equiv (0.31 g) of pyridine were dissolved in 40 mL of freshly distilled THF in a flame-dried, N_2-flushed addition funnel. The solution was added dropwise over 30 min to a solution of crotonyl chloride (3.8 mmol, 1.5 equiv) in 25 mL of THF. The reaction mixture became cloudy, and a precipitate formed on the walls. The reaction mixture was stirred under a N_2 atmosphere overnight. The THF was removed by rotary evaporation, and the residue was dissolved in chloroform. The resulting solution was washed with water (3 × 25 mL), dilute aqueous sodium bicarbonate (3 × 25 mL), and brine, whereupon it was dried over anhydrous Na_2SO_4, and the solvent was removed by rotary evaporation to yield an oil. Crude product (0.75 g) was purified by flash chromatography (silica gel, 15 vol % ethyl acetate in hexanes; **2** $R_f = 0.29$; **11** $R_f = 0.35$) to yield 0.29 g (38%) of **2** as a white solid. A portion was recrystallized from methylene chloride/hexane to give fine white needles. Mp: 80 °C. ^{1}H NMR ($CDCl_3$): δ (ppm) 1.89, (dd, $J = 6.6$, 1.5 Hz, CH_3), 3.27 (t, $J = 6.3$ Hz, CH_2S), 3.83 (s, OCH_3), 4.32 (t, $J = 6.6$ Hz, CH_2O), 6.15 (dd, $J = 15.6$, 1.5 Hz, C*H*CHCH$_3$), 6.29 (d, $J = 15.9$ Hz, CHC*H*CO), 6.90 (dd, $J = 6.6$, 2.1 Hz, aromatic C(3)*H*, C(5)*H*), 6.9−7.0 (m, CHC*H*CH$_3$), 7.47 (dd, $J = 7.0$, 2.1 Hz, aromatic C(2)*H*, C(6)*H*), 7.65 (d, $J = 15.9$ Hz, C*H*CHCOO). Anal. Calcd for $C_{16}H_{18}O_4S$: C, 62.73; H, 5.92. Found: C, 62.71; H, 5.96. UV λ_{max} 310 nm ($\epsilon = 2.23 \times 10^4$ M^{-1} cm^{-1}).

A.5. *S*-Sorboyl-2-mercaptoethyl 4-methoxycinnamate (4). Preparation was analogous to that described above for **2**, with sorboyl chloride substituted for crotonyl chloride. Flash chromatography on silica gel with 15 vol % of ethyl acetate in hexanes ($R_f = 0.18$) gave a white solid (32% after chromatography). A portion of this was recrystallized from methylene chloride/hexane to give a fine white solid. Mp: 74−76 °C. ^{1}H NMR ($CDCl_3$): δ (ppm) 1.86 (d, $J = 5.1$ Hz, CH_3), 3.27 (t, $J = 4.8$ Hz, CH_2S), 3.82 (s, OCH_3), 4.31 (t, $J = 4.8$ Hz, CH_2O), 6.0−6.3 (m, 3H, sorboyl), 6.28 (d, $J = 11.7$ Hz, CHC*H*CO), 6.88 (d, $J = 6.6$ Hz, 2H, aromatic C(3)*H*, C(5)*H*), 7.15−7.24 (m, 1H, sorboyl), 7.47 (d, 2H, aromatic C(2)*H*, C(6)*H*), 7.6 (d, $J = 12.3$ Hz, 1H, C*H*CHCO). Anal. Calcd for $C_{18}H_{20}O_4S$: C, 65.04; H, 6.06. Found: C, 64.95; H, 5.99. UV λ_{max} 228 nm ($\epsilon = 1.64 \times 10^4$ M^{-1} cm^{-1}), λ_{max} 294 nm ($\epsilon = 4.23 \times 10^4$ M^{-1} cm^{-1}).

A.6. *N,N′*-(Dithiodiethylene)bis(*p*-methoxycinnamide) (8). Cystamine dihydrochloride (8.0 g, 0.0356 mol) was converted to the free base by treatment with 2 equiv of aqueous sodium hydroxide followed by extraction with chloroform. The chloroform solution was then dried with Na_2SO_4. To this chloroform solution were added **7** (7.0 g, 0.0356 mol) and triethylamine (7.12 g, 0.0712 mol), and the solution was refluxed for 2.5 h. The solution was washed twice with water, twice with dilute HCl, once with dilute $NaHCO_3$, once with brine, and dried (Na_2SO_4), and the solvent was removed by rotary evaporation to give a tan solid (6.75 g, 80%). Recrystallization from ethanol gave a white solid. Mp: 263−265 °C. ^{1}H NMR (DMSO-d_6): δ (ppm) 2.84 (t, $J = 6.6$ Hz, 2H, CH_2S), 3.46 (dt, $J = 6.6$, 6.3 Hz, 2H, NCH_2), 3.75 (s, 3H, OCH_3), 6.47 (d, $J = 15.9$ Hz, C*H*CO), 6.93 (d, $J = 9.0$ Hz, 2H, aromatic C(3)*H*, C(5)*H*), 7.37 (d, $J = 15.9$ Hz, 1H, COCHC*H*), 7.48 (d, $J = 8.7$ Hz, 2H, aromatic C(2)*H*, C(6)*H*), 8.22 (t, $J = 5.9$ Hz, 1H, N*H*).^{13}C NMR (DMSO-d_6): δ (ppm) 37.4, 38.1, 55.2, 114.4, 119.4, 127.4, 129.1, 138.6, 160.3, 165.4.

A.7. 2-Mercaptoethyl *p*-methoxycinnamide (9). Preparation of **9** was generally carried out as described for **11**, except the solvent was 10% acetone in ethanol. Disulfide **8** (1.65 g, 0.0035 mol) in 150 mL of ethanol with 20 mL of acetone was treated with 3.5 g of zinc powder, 15 mL of glacial acetic acid, and 8 mL of concentrated HCl. After 1 h, the reaction was worked up as described for **11** to yield 1.25 g (76%). ^{1}H NMR ($CDCl_3$): δ (ppm) 1.39 (t, $J = 8.1$ Hz, S*H*), 2.75 (dt, $J = 6.6$, 8.1 Hz, CH_2SH), 3.57 (dt, $J = 6.6$, 6.0 Hz, CH_2NH), 3.82 (s, OC*H*), 6.28 (d, $J = 15.6$ Hz, C*H*CO), 5.95 (br s, N*H*), 6.89 (d, $J = 9.0$ Hz, aromatic C(3)*H*, C(5)*H*), 7.46 (d, $J = 8.7$ Hz, aromatic C(2)*H*, C(6)*H*), 7.60 (d, $J = 15.3$ Hz, C*H*CHCO).

A.8. *S*-Crotonyl-2-mercaptoethyl 4-methoxycinnamide (1). Compound **1** was prepared from thiol **9** following the procedure described above for compound **2**. Crude product was purified by flash chromatography (silica gel, 30 vol % acetone in hexanes; **1** $R_f = 0.23$) to give **1** as a white solid in 24% yield. Recrystallization from methylene chloride/hexane gave a white solid. Mp: 107−109 °C. ^{1}H NMR ($CDCl_3$): δ (ppm) 1.89 (dd, $J = 7.1$, 1.7 Hz, CH_3), 3.16 (t, $J = 6.6$ Hz, CH_2S), 3.60 (dt, $J = 6.6$, 5 Hz, NHCH_2), 3.83 (s, OCH_3), 6.04 (br s, N*H*), 6.16 (dd, $J = 14$, 1.8 Hz, C*H*CHCH$_3$), 6.23 (d, $J = 15.3$ Hz, CHC*H*CONH), 6.88 (dd, $J = 6.9$, 21.8 Hz, aromatic C(3)*H*, C(5)*H*), 6.9−7.0 (m, CHC*H*CH$_3$), 7.45 (d, $J = 8.4$ Hz, aromatic C(2)*H*, C(6)*H*), 7.56 (d, $J = 16.2$ Hz, C*H*CHCOO). ^{13}C NMR ($CDCl_3$): δ (ppm) 18.0, 28.3, 40.0, 47.6, 55.3, 114.2, 118.0, 127.5, 129.4, 129.9, 140.9, 141.8, 160.9, 166.5. Mp: 107−109 °C. Anal. Calcd for $C_{16}H_{19}NO_3S$: C, 62.93; H, 6.27; N, 4.59. Found: C, 62.74; H, 6.31; N, 4.51. UV λ_{max} 224 nm ($\epsilon = 3.00 \times 10^4$ M^{-1} cm^{-1}), λ_{max} 292 nm ($\epsilon = 2.87 \times 10^4$ M^{-1} cm^{-1}).

A.9. *S*-Sorboyl-2-mercaptoethyl 4-methoxycinnamide (3). Compound **3** was prepared from thiol **9** following the procedure described above for compound **2**. After 4 h, the reaction was judged complete by TLC, and the reaction mixture was worked up as described for compound **2** to yield a brown oil. Crude product was purified by flash chromatography (silica gel, 30 vol % acetone in hexanes; **3** $R_f = 0.34$). The product was obtained as a white solid in 40% yield and was then twice recrystallized from methylene chloride/hexane. Mp: 128−129 °C. ^{1}H NMR ($CDCl_3$): δ (ppm) 1.87 (d, $J = 6.0$ Hz, CHCH_3), 3.17 (t, $J = 6.0$ Hz, CH_2S), 3.61 (t, $J = 5.4$ Hz, NHCH_2), 3.82 (s, OCH_3), 6.0−6.25 (m, 3H, sorboyl C*H*s), 6.24 (d, $J = 15.6$ Hz, CHC*H*CONH), 6.87 (d, $J = 9.0$ Hz, aromatic C(3)*H*, C(5)*H*), 7.16−7.28 (m, 1H, sorboyl C*H*), 7.43 (d, $J = 9.0$ Hz, aromatic C(2)*H*, C(6)*H*), 7.56 (d, $J = 15.3$ Hz, C*H*CHCONH). Anal. Calcd for $C_{18}H_{21}O_3NS$: C, 65.23; H, 6.39; N, 4.23. Found: C, 65.11; H, 6.38; N, 4.18. UV λ_{max} 224 nm ($\epsilon = 1.80 \times 10^4$ M^{-1} cm^{-1}), λ_{max} 292 nm ($\epsilon = 4.52 \times 10^4$ M^{-1} cm^{-1}).

A.10. Adduct of 2 and *N*-acetylcysteamine (16). Synthesis of compound **16** was carried out on a 40-mg scale. Compound **2** (40 mg) in $CHCl_3$ was treated with 1 equiv of NAC and 0.1 equiv of DBN. After 30 min, the product was purified by chromatography (silica, 40% ethyl acetate/hexane to 100% ethyl acetate) to give **16** in 53% yield. ^{1}H NMR ($CDCl_3$): δ (ppm) 1.30 (d, $J = 6.6$ Hz, SCHCH_3), 1.98 (s, O═CCH_3), 2.66 (t, $J = 6.3$ Hz, NHCH$_2$CH_2S), 2.7−2.8 (m, O═CCH_2), 3.2−3.3 (m, SC*H*CH$_3$ and CH$_2$CH_2SCO), 3.41 (m, NHCH_2CH$_2$S), 3.81 (s, OCH_3), 4.29 (t, $J = 6.6$ Hz, OCH_2-CH$_2$S), 6.1 (br s, N*H*), 6.27 (d, $J = 15.9$ Hz, CH═C*H*CO), 6.88 (d, $J = 8.1$ Hz, aromatic C(3)*H*, C(5)*H*), 7.45 (d, $J = 8.1$ Hz, aromatic C(2)*H*, C(6)*H*), 7.63 (d, $J = 15.9$ Hz, C*H*═CHCO). ^{13}C NMR ($CDCl_3$): δ (ppm) 21.6 (O═C*C*H$_3$), 23.2 (SCH*C*H$_3$), 28.0

(OCH_2CH_2S), 30.6 (SCH_2CH_2NH), 36.3 ($SCHCH_3$), 38.8 (CH_2-NH), 51.0 (O═CCH_2), 55.4 (OCH_3), 62.5 (OCH_2CH_2S), 114.4 (aromatic *C*(3), *C*(5)), 114.9 (CHCHCOO), 127.0 (aromatic *C*(1)), 129.8 (aromatic *C*(2), *C*(6)), 145.1 (*C*HCHCOO), 161.5 (aromatic C(4)), 166.9 (CHCH*C*OO), 170.2 (NH*C*O), 196.5 (S*C*O). COSY, HMBC, and HMQC spectra are shown in the Supporting Information.

B. Syntheses of Anthranilates. B.1. Bis(anthranilate ester) of hydroxyethyl disulfide (12). A solution of isatoic anhydride (15.0 g, 0.092 mol), hydroxyethyl disulfide (7.08 g, 0.0459 mol), and DMAP (1.11 g, 0.0091 mol) in DMF was heated under N_2 at 95 °C overnight. The DMF was removed by rotary evaporation at 60 °C, and the brown liquid obtained was diluted with chloroform. The resulting solution was washed twice with water, once with brine, and dried with Na_2SO_4. The solvent was removed by rotary evaporation to give a brown oil. Crystallization occurred either spontaneously or upon dilution with 100 mL of methanol followed by trituration with water (50 mL). The precipitate was collected by filtration, washed with methanol, and air-dried to give an off-white solid, 17.0 g (94%). Mp: 90−92 °C. TLC $R_f = 0.75$ (silica, hexane/acetone, 1:1, v/v). ^{1}H NMR ($CDCl_3$): δ (ppm) 3.07 (t, J = 6.6 Hz, 2H, CH_2O), 4.54 (t, J = 6.6 Hz, CH_2S), 5.5 (br s, NH_2), 6.61−6.66 (m, 2H, C(3)*H*, C(5)*H*), 7.26 (ddd, J = 8.3, 6.9, 1 Hz, 1H, C(4)*H*), 7.86 (d, J = 8.1 Hz, 1H, C(6)*H*). ^{13}C NMR ($CDCl_3$): δ (ppm) 37.0, 61.6, 110.0, 115.8, 116.2, 130.8, 133.8, 150.1, 167.3.

B.2. Bis(*N*-methylanthranilate ester) of hydroxyethyl disulfide (13). Preparation of **13** from *N*-methylisatoic anhydride was carried out as described for **12**. Crude product was obtained as a brown liquid, which solidified somewhat upon standing. The crude product was triturated with methanol, collected by filtration, and washed with methanol to give a tan solid. Mp: 67−69 °C. A second crop was obtained by dropwise addition of water to the methanol filtrate. ^{1}H NMR ($CDCl_3$): δ (ppm) 2.91 (s, 3H, NCH_3), 3.06 (t, J = 6.6 Hz, 2H, SCH_2), 4.53 (t, J = 6.6 Hz, 2H, CH_2O), 6.60 (t, J = 7.4 Hz, 1H, C(5)*H*), 6.69 (d, J = 9.0 Hz, 1H, C(3)*H*), 7.39 (ddd, J = 7.8, 7.5, 1.8 Hz, C(4)*H*), 7.8 (br s, 1H, N*H*), 7.91 (dd, J = 7.8, 1.5 Hz, C(6)*H*).

B.3. 2-Mercaptoethyl anthranilate (14). Disulfide **12** (8.20 g, 0.021 mol) was slowly added to 500 mL of warm absolute ethanol, and it dissolved with stirring. The solution was then cooled to 20 °C and was treated with 2 mL of glacial acetic acid, 25.4 g of zinc powder, and 8 mL of concentrated HCl (added in portions). The solution was stirred for 3 h, at which time the reaction was judged to be complete by TLC (silica, 20% acetone in hexanes; disulfide $R_f = 0.18$, thiol $R_f = 0.45$). The reaction mixture was then filtered, and the ethanol was largely removed in vacuo. The remaining syrup was dissolved in chloroform, and the organic phase was washed with water, dilute aqueous bicarbonate, saturated aqueous bicarbonate, and finally with brine. The organic fraction was dried (Na_2SO_4), and the solvent was removed in vacuo to give a syrup (2.37 g, 69%). ^{1}H NMR ($CDCl_3$): δ (ppm) 1.6 (br s, S*H*), 2.85 (t, J = 6.6 Hz, CH_2S), 4.38 (t, J = 6.6 Hz, CH_2O), 5.64 (br s, NH_2), 6.6−6.7 (m, 2H, aromatic C(3)*H*, C(5)*H*), 7.26 (ddd, J = 6.9, 6.9, 1.8 Hz, C(4)*H*), 7.87 (dd, J = 8.1, 1.1 Hz, C(6)*H*). ^{13}C NMR ($CDCl_3$): δ (ppm) 23.3, 65.4, 110.2, 116.1, 116.6, 131.0, 134.1, 150.4, 167.5.

B.4. 2-Mercaptoethyl *N*-methylanthranilate (15). Disulfide **13** (4.1 g, 0.0097 mol) was dissolved in 100 mL of acetonitrile, and the solution was chilled in an ice bath. To the solution were added in portions 18 g of zinc powder, 4 mL of glacial acetic acid, and a total of 7 mL of concentrated HCl. After 2 h, reduction was judged to be complete by TLC (silica, 20% acetone in hexanes; disulfide $R_f = 0.41$, thiol $R_f = 0.56$). After filtration to remove solids, the solvent was removed in vacuo, and the residue was dissolved in chloroform. The resulting solution was washed with water, very dilute aqueous bicarbonate, very dilute sodium hydroxide (gently to avoid emulsion), and finally brine, whereupon it was dried with anhydrous Na_2SO_4. The chloroform was removed in vacuo, and the thiol was obtained as an oil (2.91 g, 71%), which was stored under N_2 at 4 °C. A small portion was purified by crystallization in methylene chloride/hexane and then sublimation to yield fluffy, light yellow florettes. Mp: 33−35 °C. ^{1}H NMR ($CDCl_3$): δ (ppm) 1.56 (t, J = 8.7 Hz, S*H*), 2.86 (t, J = 7.4 Hz, 2H, CH_2S), 2.92 (s, N−CH_3), 4.38 (t, J = 6.6 Hz, 2H, CH_2O), 6.66 (dd, J = 15, 1.2 Hz, 1H, C(5)*H*), 6.72 (d, J = 8.4 Hz, C(3)*H*), 7.43 (dd, J = 8.0, 1.5 Hz, 1H, C(4)*H*), 7.92 (dd, J = 8.4, 1.5 Hz, 1H, C(6)*H*).

B.5. *S*-Crotonyl-2-mercaptoethyl *N*-methylanthranilate (5). The reaction was carried out under an argon atmosphere. Thiol **15** (3.52 g, 16.7 mmol) was dissolved in 80 mL of THF, and crotonic anhydride (2.57 g, 16.7 mmol) was added, followed by 0.40 g (16.7 mmol) of NaH, added in portions. Vigorous H_2 evolution was observed. Additional portions of crotonic anhydride and NaH were added until TLC showed that thiol was totally consumed. After 1.5 h, the solvent was removed by rotary evaporation not quite to dryness, and the residue was diluted with water and extracted twice with ether. The organic phase was washed with dilute aqueous $NaHCO_3$ followed by brine, and then it was dried with Na_2SO_4. Removal of solvent in vacuo gave 4.92 g of crude product. Purification by flash chromatography (silica, 10 vol % ethyl acetate in hexanes) yielded 2.26 g of **5** (48%) as a white solid. Mp: 42−43 °C. ^{1}H NMR ($CDCl_3$): δ (ppm) 1.89 (dd, J = 7.2, 1.8 Hz, COCHCHCH_3), 2.91 (s, NCH_3), 3.32 (t, J = 6.6 Hz, CH_2S), 4.39 (t, J = 6.6 Hz, CH_2O), 6.15 (dd, J = 15.6, 1.7 Hz, C*H*CHCH$_3$), 6.63 (dd, J = 7.8, 6.6 Hz, aromatic C(5)*H*), 6.73 (d, J = 8.1 Hz, C(3)*H*), 6.94 (dq, J = 15.3, 8.1 Hz, CHC*H*CH$_3$), 7.40 (ddd, J = 8.1, 7.8, 1.5 Hz, C(4)*H*), 7.90 (dd, J = 8.2, 1.5 Hz, C(6)*H*). Anal. Calcd for $C_{14}H_{17}NO_3S$: C, 60.19; H, 6.13; N, 5.01. Found: C, 60.28; H, 6.20; N, 5.02. UV λ_{max} 255 nm ($\epsilon = 1.55 \times 10^3$ M^{-1} cm^{-1}), λ_{max} 357 nm ($\epsilon = 6.96 \times 10^3$ M^{-1} cm^{-1}).

B.6. *N,S*-Di(crotonyl)-2-mercaptoethyl anthranilate (6). The reaction was carried out in N_2-flushed glassware. A solution of **14** and 2.9 equiv of pyridine in dry THF was added via addition funnel at room temperature to a stirred solution of 2.9 equiv of crotonyl chloride in THF. After 3 h, the reaction mixture had a copious solid and was reddish−brown in color. The reaction was complete as judged by the absence of thiol **14** by TLC. The reaction mixture was worked up as described for compound **2** to give a reddish oil in 89% yield. Flash chromatography (silica gel, 20% ethyl acetate in hexanes) gave a white solid (36% yield). Mp: 66−68 °C. ^{1}H NMR ($CDCl_3$): δ (ppm) 1.89 (dd, J = 7.1, 1.8 Hz, SCOCH-CHCH_3), 1.92 (dd, J = 7.2, 1.8 Hz, NHCOCHCHCH_3), 3.34 (t, J = 6.6 Hz, CH_2S), 4.45 (t, J = 6.3 Hz, CH_2O), 6.02 (dd, J = 15.3, 1.5 Hz, NHCOC*H*CHCH$_3$), 6.16 (dd, J = 15.5, 1.8 Hz, SOC*H*-CHCH$_3$), 6.90−7.10 (m, 3H, aromatic C(5)H, NHCOCHC*H*CH$_3$ and SOCHC*H*CH$_3$), 7.54 (ddd, J = 7.8, 8.1, 1.2 Hz, C(4)*H*), 8.02 (dd, J = 8.7, 1.7 Hz, C(6)*H*), 8.79 (d, J = 8.7 Hz, aromatic C(3)-*H*), 11.1 (br s, N*H*). ^{13}C NMR ($CDCl_3$): δ (ppm) 17.8, 17.9, 27.1, 63.5, 114.5, 120.3, 122.2, 126.6, 129.6, 130.8, 134.7, 141.1, 141.8, 141.9, 164.3, 167.8, 188.5. Recrystallization was from methylene chloride/hexanes. Anal. Calcd for $C_{17}H_{19}NO_4S$: C, 61.24; H, 5.74; N, 4.20. Found: C, 61.19; H, 5.75; N, 4.26. UV λ_{max} 264 nm ($\epsilon = 1.75 \times 10^4$ M^{-1} cm^{-1}), λ_{max} 315 nm ($\epsilon = 9.65 \times 10^3$ M^{-1} cm^{-1}).

C. Time-Course NMR Spectral Study of Reaction of 2 and 4 with *N*-Acetylcysteamine. A solution of 17 mg of **2** (0.055 mmol) was dissolved in 1 mL of $CDCl_3$, and the ^{1}H NMR spectrum was recorded. One equivalent of *N*-acetylcysteamine was added, and the spectrum was recorded. It showed that no reaction had occurred. One-tenth equivalent of the base DBN (1,5-diazabicyclo[4.3.0]non-5-ene) was added, and the NMR spectrum was recorded within 2 min. A COSY spectrum confirmed the structure of the adduct that had formed. An analogous NMR study with **4** was carried out as described above, except NMR spectra were recorded at times up to 15 min after DBN addition.

D. HPLC Kinetics of Conjugate Addition. HPLC was carried out with a reverse phase C18 column (300 × 3.9 mm) with isocratic elution with acetonitrile/water (1:1, v/v) as the mobile phase. UV detection was used (310 nm). Data reported are the average of at least two determinations. The retention times of **1** and **3** were 4.6 and 7.2 min, respectively.

The structures of the adducts formed by the reaction of **1** with *N*-acetyl-L-cysteine and N^2-acetyl-L-lysine in the HPLC kinetics experiments were confirmed by mass spectrometry (MS) and NMR spectroscopy (^{1}H and COSY; Supporting Information). By MS (MALDI), the reaction mixture of **1** and *N*-acetyl-L-cysteine showed *m*/*z* 469.135 (calcd for $[M + H]^+$ 469.146) and 491.124 (calcd for $[M + Na]^+$ 491.128). For NMR spectroscopy, the *N*-acetyl-L-cysteine reaction was carried out in a 1:1 (v/v) mixture of 50 mM NaH_2PO_4 in D_2O (pD 8.4) and acetonitrile-d_3. Addition of the thiol group of *N*-acetyl-L-cysteine to the crotonyl C═C was evidenced by the disappearance of the vinyl resonances of **1** and the upfield shift of the crotonyl methyl group from δ 1.75 to 1.17 ppm. The COSY spectrum revealed that the new CH_3 signal was coupled to the multiplet at δ 3.2 ppm, which is due to the proton on the crotonyl C-3 that is attacked by the thiol. In the case of N^2-acetyl-L-lysine, addition was carried out in a 1:1 (v/v) mixture of 50 mM sodium borate in D_2O (pD 10.2) and acetonitrile-d_3. Spectral changes analogous to those seen with *N*-acetyl-L-cysteine were observed. The spectra of both adducts reflect the complexity resulting from the formation of diastereomers (*E* and *Z* rotamers at the amide linkage, *R* and *S* configurations at crotonyl C-3, and either *R* configuration at C-2 of the reactant *N*-acetyl-L-cysteine or *S* configuration at C-2 of the reactant N^2-acetyl-L-lysine). Contributing to the complexity is the presence of diastereotopic hydrogens in the methylene groups. Coupling of the crotonyl C-2 hydrogens to the C-3 hydrogen was not observed for either adduct, as the former protons exchange with deuterium from solvent.

As shown in Scheme 3, the preequilibrium involves attack of a dianion (carboxylate and thiolate) on a neutral species (thiol ester), and the rate-limiting step involves protonation of the resulting dianion (carboxylate and enolate) by either neutral (zwitterionic) HEPES or water. To a first approximation, according to the theory of salt effects on reaction rates, neither step should experience a salt effect (i.e., dependence of reaction rate on $z_A z_B \Gamma^{1/2}$, where z_A and z_B are the charges on the reactants and Γ is the ionic strength) because $z_A = 0$. This, and the use of HPLC for analysis, justified the omission of inert salt to maintain constant ionic strength.

It was found to be convenient to determine pH values in reaction mixtures from a pH calibration curve, created by measurement of pH in the aqueous solution (pH_{Aq}) containing either 72 mM sodium HEPES and 50 mM NAC or 154 mM sodium borate and 28.6 mM NAL, followed by addition of an equal volume of acetonitrile and then measurement of the pH of the water/acetonitrile solution (pH_{meas}). Addition of 0.18 to that value produced pH_{AqACN}. A plot of pH_{AqACN} (ordinate) versus pH_{Aq} (abscissa) was fitted with an arbitrary function to allow intermediate values of pH_{Aq} used for kinetic runs to be converted to pH_{AqACN} by calculation, without the need for delaying acquisition of kinetics data by a pH measurement or exposure of the kinetics solution to the liquid-junction salt bridge of the pH electrode. The deviation of calculated values of pH_{AqACN} did not in any case deviate from the actual values of pH_{AqACN} used for creation of the calibration curve by more than 0.07 units and typically by only 0.02 units or less. The possible influence of varying HEPES (Figure 4) from 72 mM or NAC (Figure 5) from 50 mM was neglected.

E. Curve Fitting of Kinetics Data. Curve fitting of the data in Figures 3−5 and 7 was by the equally weighted, linear least-squares method. Slope and *y*-intercept of the line in Figure 4 are reported ± their standard errors, and uncertainties in kinetic parameters derived from values of slope and intercept were calculated on the basis of propagation of errors by differential error analysis. The line in Figure 5 was constrained to have a zero *y*-intercept, in accord with eq 3. If an unconstrained fit is carried out, the slope is 2.79×10^{-1} M^{-1} min^{-1} and the *y*-intercept is (the physically meaningless) -5.89×10^{-4} min^{-1}, with $r^2 = 0.995$. For the curves in Figure 6, an equation of the form of eq 3b was fitted to the data by minimization of the sum of the squared deviations of calculated log k_{obs} from experimental values of log k_{obs}, with the ratio of rate constants k_{HEP}/k_0 set equal to 41 M^{-1}, as determined from Figures 4 and 5. The curve in Figure 8 is line-of-sight.

For determination of pK_a values, the method of half-neutralization was employed. The apparent pK_a of HEPES under our reaction conditions of 1:1 (v/v) H_2O/acetonitrile was determined by treatment of 5.0 mL of 72 mM HEPES in water, containing a 1:1 mixture of zwitterion and anion by adjustment to pH 7.54,[43] with 5.0 mL of acetonitrile, and measurement of the pH.[44] Values of pH meter readings from duplicate runs were 7.61 and 7.63. These values, as well as the other pH meter readings in 1:1 (v/v) water/acetonitrile, were converted to actual pH values in 1:1 water/acetonitrile by addition of 0.18 to the meter reading,[45] giving pK_a = 7.80 for HEPES in 1:1 (v/v) water/acetonitrile, which is reasonable, relative to the value of 7.91 found in 50% aqueous acetone.[46] The apparent pK_a of *N*-acetyl-L-cysteine in 1:1 (v/v) water/acetonitrile was determined by treatment of 4.0 mL of 50 mM *N*-acetyl-L-cysteine in water, containing a 1:1 mixture of thiol and thiolate by adjustment to pH 9.52,[47] with 4.0 mL of acetonitrile, and measurement of the pH. After addition of 0.18 to the pH meter reading, the pK_a value of 10.77 resulted. By comparison, the pK_a of mercaptoethanol similarly shifts from 9.5 in water[48] to 10.8 in 1:1 acetonitrile/water,[49] and the pK_a of methyl mercaptopropanoate shifts from 9.3 in water[48] to 10.7 in 1:1 acetonitrile/water.[49]

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Supporting Information Available: General experimental methods and NMR spectra of synthetic intermediates, thiol esters, and adducts with *N*-acetylcysteamine, *N*-acetyl-L-cysteine, and N^2-acetyl-L-lysine are presented, as well as UV absorbance spectra of compounds **1**−**6**. This material is available free of charge via the Internet at http://pubs.acs.org.

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