Selenol Esters as Specific Reagents for the Acylation of Thiol Groups[1,2]

Alexandros Makriyannis, Wolfgang H. H. Günther, and Henry G. Mautner*

Contribution from the Department of Biochemistry and Pharmacology, Tufts University School of Medicine, Boston, Massachusetts 02111.
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Abstract: Choline selenol esters have been found to react, in excellent yield, under mildly acidic conditions with thiols to form thiol esters. These agents do not react with amino groups under these conditions and can, therefore, be used for selectively labeling thiol groups in molecules of biological interest. The reaction of benzoyl selenol choline with choline thiol anion was found to have $K_{eq} \sim 1000$ and $k_2 \sim 4 \times 10^4\ M^{-1}\ min^{-1}$. The synthetic usefulness of choline selenol esters is outlined.

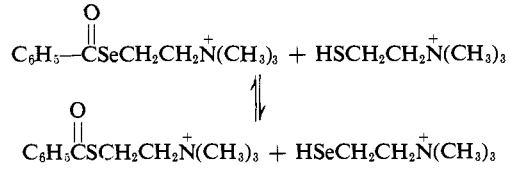
Thiol groups play a vital role in the functions of a large number of biopolymers. The active sites of enzymes, subunit interactions, and certain membrane functions may involve mercapto groups. For this reason, numerous attempts have been made to develop highly specific thiol group reagents. Such reagents are useful in determining whether the thiol group is crucial for the functioning of the biopolymer (as, for instance, in the case of papain).[3] They have also been used to introduce fluorescent or other groupings[4] as tracers of the thiol group microenvironment.

Unfortunately, the previous use of such reagents has encountered certain practical problems. Many of the compounds used for labeling thiol groups may also react with amino groups. Furthermore, some reagents, such as *N*-ethylmaleimide and its derivatives, form reaction products from which the labeling group cannot be removed once it is attached.

We noted some time ago that selenol esters react smoothly with thiols to yield thiol esters.[5,6] Selenol esters also react, although very slowly, with disulfides to form thiol esters; in the latter case, acylation is preceded by reduction.[7] These reactions appeared of interest for several reasons. Selenol esters are capable of reacting with thiols at pH's at which amino groups are protonated. For that reason, selenol esters in slightly acidic solutions are capable of acylating the thiol groups of peptides selectively while lysine and histidine residues remain unreactive. The ultraviolet absorption maxima of selenol esters differ from those of the corresponding thiol esters. The reaction of selenol esters with thiols to form thiol esters can, therefore, be followed very conveniently, since the extinction maxima of reactant and of product can be monitored simultaneously at different wavelengths. The thiol esters are particularly convenient thiol derivatives because of their low susceptibility to hydrolysis[8-10] and extremely high susceptibility to aminolysis.[11,12] Thiol esters, when used as protective groups, are quite stable under normal experimental conditions and may be easily removed by the addition of free amine.[13]

While investigating the usefulness of selenol esters as reagents for acylating thiols of biological importance, a series of compounds containing the trimethylammonium group was synthesized. Selenol esters such as acetyl selenol choline,[14] benzoyl selenol choline,[14] and their derivatives[15] are highly water soluble and may readily be removed from reaction mixtures by the use of ion-exchange chromatography or gel filtration.

In the present investigation we examined the reaction



with the intention of determining the equilibrium constant, as well as the enthalpy of activation and entropy of activation. Since the reactant and the resulting acyl compounds are identical, except for the substitution of sulfur for selenium, interpretation of the results is relatively free of complications introduced by steric factors. In view of the importance of buffer effects in the hydrolysis of benzoyl selenol choline,[16] the dependence of the thiolysis on ionic strength and buffer concentration was examined. The reaction rates and relative reactivities of benzoylselenol choline with various thiols of biological interest were also obtained. Finally the usefulness of benzoyl selenol choline as a reagent for the synthesis of amino acid or peptide thiol esters was investigated.

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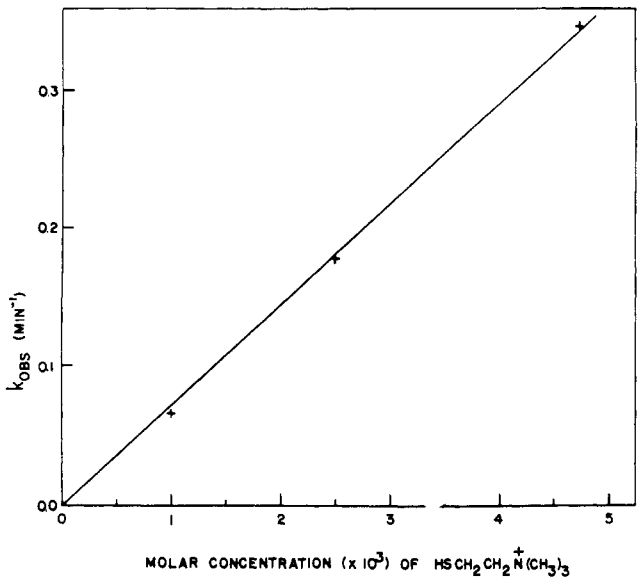

Figure 1. The effect of choline thiol concentration on the rate of the reaction with 10^{-4} *M* benzoyl selenol choline iodide in 0.1 *M* phosphate buffer (pH 5.0) at 30°. The ionic strength was 1.0. Each point represents the average of three determinations.

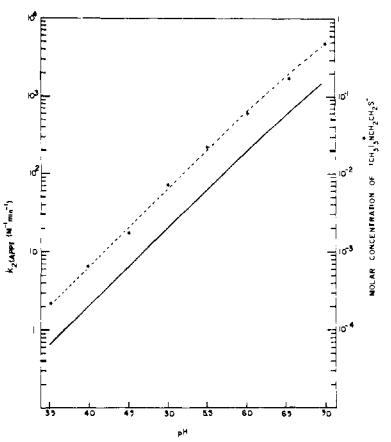

Figure 2. (----) pH dependence of the reaction of choline thiol iodide (2.5×10^{-3}, 5×10^{-3}, or 5×10^{-2} *M*) and benzoyl selenol choline iodide (2.5×10^{-4} *M*) in 0.1 *M* phosphate buffer (pH 5.0) at 30°. The apparent rate constants (k_2(app)) were calculated from the observed pseudo-first-order rate constants. The ionic strength was 1.0. (——) pH dependence of the concentration of ionized thiol calculated using a pK_a of 7.7 for choline thiol. The intrinsic second-order rate constant based on these concentrations was $3.6 \times 10^4\ M^{-1}\ \text{min}^{-1}$.

Experimental Section

Materials. L-(−)-Cysteine hydrochloride, DL-serine, DL-lysine monohydrochloride, DL-homocysteine, and glutathione were commercial products. Coenzyme A was purchased from P-L Biochemicals, Inc. Thiol choline, benzoyl thiol choline, choline diselenide, benzoyl selenol choline, anisoyl thiol choline, and anisoyl selenol choline were synthesized according to literature methods.[14] Each choline derivative was recrystallized at least twice. Phosphate buffers were utilized throughout all of the kinetic measurements. The ionic strength was maintained with sodium chloride. Stock solutions were prepared on the day on which they were used. All reagent and buffer solutions were thoroughly flushed with argon.

Methods. Equilibrium Constants. Spectrophotometric Method. The equilibrium constants for the benzoyl transfer reaction were determined in a 0.1 *M* phosphate–hypophosphite (1:1) buffer, at 30.0 ± 0.5° by measuring the decreases in absorption in the region of 285 nm at a constant pH with a Cary Model 15 recording spectrophotometer equipped with a thermostated cuvette compartment. Each measurement was initiated by the addition of an aliquot from a temperature-equilibrated solution of choline thiol to a temperature-equilibrated cuvette containing the benzoyl selenol choline and buffer equipped with a tight-fitting Teflon stopper. Mixing of the reagents was effected by bubbling a slow stream of argon through the reaction mixture. The change in absorbance with time was followed until absorbance remained constant for at least 10 min. The reaction was then driven to completion by the addition of an aliquot equivalent to a 100-fold excess of thiol solution and the change in absorbance was recorded. The measured absorbance at 285 nm was corrected for the absorption of the excess thiol added. The concentrations of reactants and products at equilibrium were then calculated from absorbance measurements.

Equilibrium Constants. Nuclear Magnetic Resonance (Nmr) Method. (A) Determined from the Reaction of Choline Thiol with Benzoyl Selenol Choline. A 5-mm nmr tube containing a solution of benzoyl selenol choline bromide (0.1 *M*) in a 1 *M* phosphate–1 *M* hypophosphite–D_2O (99.8%) buffer was placed under an atmosphere of argon. Choline thiol bromide (0.125 *M*) was then added while a slow stream of argon was being bubbled into the mixture. The tube was then sealed and allowed to stand until equilibrium was established. The final pD of the solution was measured using a glass electrode.

(B) Determined from the Reaction of Choline Selenol and Benzoyl Thiol Choline. A solution of choline diselenide dibromide (0.05 *M*) in H_3PO_2/D_2O (99.8% 1 *M*) was heated for 15 min at 75° in a 5-mm nmr tube. To the choline selenol (0.1 *M*) thus obtained, benzoyl selenol choline bromide (0.1 *M*) was added while a slow stream of argon was being bubbled into the mixture. The acidity of the solution was then adjusted by the addition of disodium phosphate (anhydrous). The tube was subsequently sealed under an atmosphere of argon and the mixture was allowed to stand until equilibrium was established. The final pD was measured using a glass electrode.

^{1}H nmr spectra at 60 MHz were obtained on a Hitachi R-20B 60-MHz high resolution spectrometer. The spectra of the mixtures at equilibrium were obtained at 34° using a sweep of 60 Hz. The equilibrium constants were calculated by examining the singlets due to the nine trimethylammonium protons. The area of each of the four singlets was used to measure the relative concentrations of the corresponding products in the reaction mixture. The results were checked by examining the nonoverlapping segments of the benzoyl selenol choline and benzoyl thiol choline aromatic multiplets.

Kinetic Measurements. The rates of the reactions of thiols with the aroyl selenol cholines were followed by observing the decrease in the benzoyl selenol choline absorption at 285 nm (305 nm in the case of anisoyl selenol choline). Measurements were made on a Cary Model 15 ultraviolet spectrophotometer as before. Cuvettes containing the aroyl selenol choline in the appropriate buffer were permitted to reach the temperatures at which the rate studies were to be conducted while a slow stream of argon was being bubbled into the mixture. Aliquots of the thiol solution were then added quickly while mixing was effected with the argon bubbler (8 sec). Temperatures inside the thermostated cuvette were determined directly by means of a calibrated thermometer. Reactions were carried out with a large excess of thiol so that the observed rates followed pseudo-first-order kinetics. The first-order rate constants, averaged from at least three determinations, were obtained from semilogarithmic plots of the difference between the absorbance at various times and the final equilibrium absorbance against time by use of the equation $k_{obsd} = 0.693/t_{1/2}$. In all cases, the plots were linear for at least 1 half-life.

***S*-Benzoylcysteine.** A mixture of L-(−)-cysteine hydrochloride (150 mg, 0.95 mmol) and benzoyl selenol choline bromide (1.20 g, 3.42 mmol) in water (4 ml) was stirred in a beaker for 24 hr. After the pH was adjusted to 6.2 using 2 *M* disodium phosphate, a white microcrystalline precipitate was obtained. The product (200 mg, 89%) was then washed and dried under vacuum: mp 142° (lit.[17] mp 142°); ir (KBr) 1660 cm^{-1} (C=O); uv λ_{max}^{pH1} 242, 264 nm (ϵ 6500, 6100); nmr (10% DCl–D_2O) δ 3.65 (doublet, J = 4.0 Hz, 1 H), 3.73 (doublet, J = 25 Hz, 1 H), 4.48 (multiplet, 1 H), 7.58 (multiplet, 3 H), 7.93 (multiplet, 2 H).

***S*-Benzoylglutathione.** A mixture of glutathione (γ-L-glutamyl-L-cysteinylglycine) (reduced) (200 mg, 0.65 mmol) and benzoyl

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selenol choline bromide (700 mg, 2.00 mmol) in water (2 ml) was stirred in a beaker for 15 hr. After the pH was adjusted to 2.8 using 2 *M* disodium phosphate, a white precipitate was obtained. The product was purified by dissolving it in 2 *N* HCl and reprecipitating it by adjusting the pH to 2.8. The white microcrystalline product (250 mg, 94%) was washed with water and dried under vacuum: mp 230° (dec); ir (KBr) 1660 cm^{-1} (C=O); uv λ_{max}^{pH1} 242, 264 nm (ϵ 10,700, 9900); nmr (10% DCl–D_2O) δ 2.34 (multiplet, 2 H), 2.70 (multiplet, 2 H), 3.58 (multiplet, 2 H), 4.08 (singlet, 2 H), 4.26 (multiplet, 1 H), 4.77 (multiplet, 1 H), 7.38–8.04 (multiplet, 5 H).

Anal. Calcd for $C_{17}H_{21}N_3O_7S$: C, 49.63; H, 5.15; N, 10.21. Found: C, 49.31; H, 5.22; N, 9.93.

***S*-Benzoyl Coenzyme A.** A mixture of benzoyl selenol choline iodide (3.51 mg, 10^{-5} mol) and coenzyme A, lithium salt (0.82 mg, 10^{-6} mol) in 0.1 *M* phosphate buffer pH 6 (1 ml), was stirred for 12 hr at 25°. *S*-Benzoyl coenzyme A was isolated in quantitative yield by gel filtration on Sephadex G-10, equilibrated, and eluted with a 0.1 *M* phosphate buffer at pH 6. The product showed identical spectroscopic characteristics with *S*-benzoyl coenzyme A as described by Gruber and Lynen.[18]

Results

Reaction Kinetics and Specificity. The reaction rate of benzoyl selenol choline with choline thiol (pK_a = 7.70)[19] to form benzoyl thiol choline and choline selenol (pK_a = 4.68)[13] showed first-order dependence on the concentration of the choline thiol anion as seen in Figures 1 and 2. Above pH 7 the reaction was too fast to be followed with the equipment available. The course of the above reaction is portrayed in Figure 3, where the gradual disappearance of the selenol ester is accompanied by the appearance of thiol ester.

In contrast to the hydrolysis of benzoyl selenol choline,[16] the thiolysis of this compound was affected neither by phosphate concentration nor by ionic strength. At pH 7.0 the reaction rate remained constant over a range of 0.05–0.2 *M* phosphate buffers. Similarly the reaction rate was not affected when the ionic strength was changed from 0.1 to 1 *M* by the addition of sodium chloride. Benzoyl selenol choline at pH 5 did not react with either histidine, lysine, or serine.

Equilibrium Constants. The thioacylation reaction was studied from both directions in deuterium oxide using proton magnetic resonance spectroscopy. In the forward reaction, benzoyl selenol choline and choline thiol were mixed in the appropriate buffer and the reaction mixture was examined after equilibrium was established. To study the reverse reaction, choline selenol was generated by the reduction of choline diselenide with hypophosphorous acid by the method of Günther.[20] Benzoyl thiol choline was then added and the reaction was initiated by adjusting the pH with sodium phosphate. In the determination of the intrinsic equilibrium constants [K_{eq}(intr)] adjustments were made for the isotope effect of the D_2O solvent. The dissociation constants of choline thiol were corrected using K_{H_2O}/K_{D_2O} values reported by Bunton and Shriner,[21] for compounds with similar dissociation constants.

Equilibrium constants for the forward reaction were also determined spectrophotometrically at two different pH's (Table I). Because of the very high oxidizability of choline selenol, all of the above studies were conducted in an argon atmosphere.

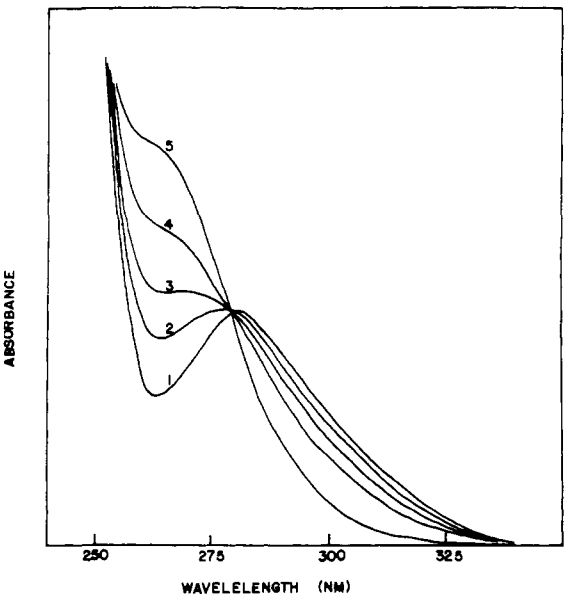

Figure 3. Time course of the reaction of benzoyl selenol choline iodide (5×10^{-5} *M*, λ_{max} 285 nm (ϵ 6700)) and choline thiol iodide (1.25×10^{-3} *M*), with the formation of benzoyl thiol choline (λ_{max} 264 nm (ϵ 9900)) and choline selenol, in 0.1 *M* phosphate buffer (pH 7.0) at 30°. The ionic strength is 1.0.

Table I. Determination of Equilibrium Constants (Benzoyl Selenol Choline + Choline Thiol ⇌ Benzoyl Thiol Choline + Choline Selenol)

Method	pH	K_{eq}(app)	ΔG(app)	K_{eq}(intr)[a]	ΔG(intr)[a]
Nmr[b]	5.30	2.39	−0.53	1059	−4.24
Nmr[c]	5.22	2.40	−0.53	1169	−4.29
Spectrophotometric[d]	7.01	140[e]	−2.96	821	−4.03
Spectrophotometric[d]	5.02	3.17[e]	−0.71	1037	−4.16

[a] For the reaction RSeCOPh + RS^- ⇌ RSe^- + RSCOPh, calculated using pK_a values of 7.7 (H_2O), 8.2 (D_2O) for choline thiol, and 4.68 (H_2O), 5.2 (D_2O) for choline selenol. [b] Calculated from the reaction of choline thiol bromide (0.125 *M*) and benzoyl selenol choline bromide (0.1 *M*) in 1 *M* phosphate–1 *M* hypophosphite buffer (D_2O) at 34°. [c] Calculated from the reaction of choline selenol bromide (0.1 *M*) and benzoyl thiol choline bromide (0.1 *M*) in 1 *M* phosphate–1 *M* hypophosphite buffer (D_2O) at 34°. [d] From the reaction of benzoyl selenol choline iodide (2.5×10^{-4} *M*) and choline thiol iodide (2.5×10^{-4} *M*) in 0.05 *M* phosphate–0.05 *M* hypophosphite buffer at 30°. The ionic strength was 1.0. [e] The average of three determinations.

Relative Reactivity of Thiol Groups. Owing to the high reactivity of the mercaptide ion with selenol esters, the reactions of cysteine, homocysteine, glutathione, and choline thiol could be followed spectrophotometrically at low pH's. Here only a very small fraction of the thiol groups is ionized. The intrinsic reactivities of these compounds were obtained by calculating the second-order rate constants of the reaction based upon the concentration of mercaptide ion (Table II). These reactivities were found to be very similar. Anisoyl selenol choline also reacted, with choline thiol (Table II), although at a slower rate.

Enthalpy of Activation, Entropy of Activation. The effects of temperature on the reaction of benzoyl selenol choline with choline thiol are summarized in Figure 4.

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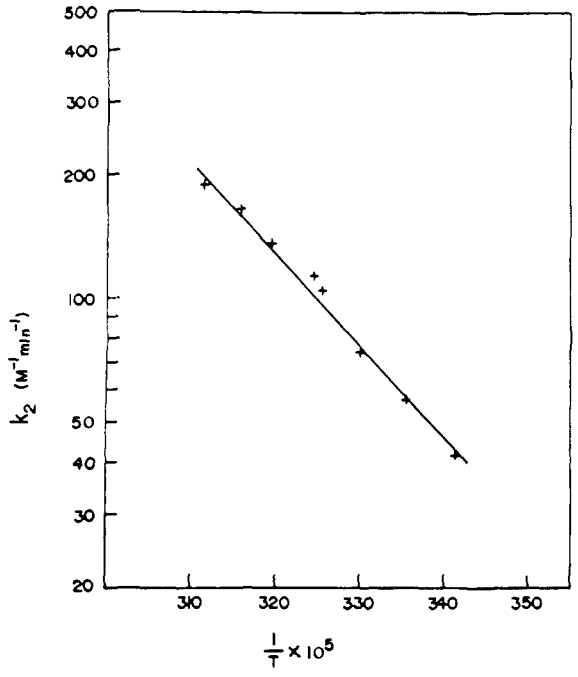

Figure 4. The effect of temperature on the rate of reaction of benzoyl selenol choline iodide (2.5×10^{-4} M) and choline thiol iodide (5×10^{-3} M) in 0.1 M phosphate buffer (pH 5.0). The ionic strength was 1.0.

Table II. Reactivity of Thiols with Aroyl Selenol Cholines[a]

Aroyl selenol choline	Thiol	Thiol concn ($\times 10^{-2}$ M)	k_2(app), M^{-1} min^{-1}	k_2(int), M^{-1} min^{-1}
Benzoyl selenol choline	Cysteine	5	1.73	3.7×10^4
Benzoyl selenol choline	Homocysteine	2.5	0.52	3.88×10^4
Benzoyl selenol choline	Glutathione	2.5	0.37	1.71×10^4
Benzoyl selenol choline	Choline thiol	0.5	72	3.05×10^4
Anisoyl selenol choline	Choline thiol	10	19.2	0.81×10^4

[a] All experiments were performed at 30° in 0.1 M phosphate buffer (pH 5.0) using an aroyl selenol choline concentration of 2.5×10^{-4} M. The ionic strength was 1.0.

From the Arrhenius plot an energy of activation of 10.08 kcal could be obtained.

The commonly used relationships

$$\Delta H^{\pm} = E_a - RT$$

$$k_2 = \frac{kT}{h} e^{\Delta S^{\pm}/R} e^{-\Delta H^{\pm}/RT}$$

yielded the following values: $\Delta H^{\pm}_{30°} = 9480$ kcal; $\Delta S^{\pm}_{app}$(pH 5) $= -26.9$ eu; $\Delta S^{\pm}_{int} = -14.5$ eu. The intrinsic value for the entropy of activation was calculated from the second-order rate constants for the above reaction without correction for the temperature dependence of pK_a.

Synthetic Applications. The use of selenol esters provides a very convenient avenue for the synthesis of amino acid or peptide thiol esters. This preparative method which involves very mild conditions is simple and highly selective. The acidic aqueous solution of the amino acid or the peptide can be treated with a three- to fivefold excess of the choline selenol esters and the mixture stirred in an open container. Under these conditions the reactivity of amino and disulfide groups is negligible. *S*-Benzoylcysteine and *S*-benzoylglutathione were prepared by this method in almost quantitative yields. Similarly, CoA was selectively S-benzoylated in quantitative yield.

Discussion

In the benzoyl selenol choline–choline thiol reaction the thiol anion most probably is the reactive species. Correspondingly, in the reverse reaction, choline selenol seems to react as the selenide anion. Equilibrium constants calculated according to this assumption agree with data obtained from the forward and the back reaction. As can be seen in Table I, under conditions where choline thiol and choline selenol are fully ionized, the equilibrium of this reaction greatly favors the formation of the thiol ester. It seems reasonable to assume that the driving force for the thiolysis of the selenol ester resides in the greater resonance stabilization of thiol esters as compared to their selenium isologs. This postulate is in accord with previous dipole moment[22] and spectroscopic investigations.[23] The measured equilibrium constant [K_{eq}(app)] decreases sharply, however, when the pH of the reaction is lowered. At pH 5 this equilibrium constant is only 3. This is due to the fact that choline selenol ($pK_a = 4.68$)[13] is a considerably stronger acid than is choline thiol ($pK_a = 7.70$).[19] Thus, at low pH's the concentration of selenol anion largely exceeds that of the thiol anion.

The effect of hydrogen ion concentration on the equilibrium of the reaction would place severe limitations for the usefulness of the selenol esters particularly as synthetic reagents. This difficulty is overcome by taking advantage of the tendency of selenols to oxidize very rapidly under conditions where thiol groups are quite stable. The equilibrium of the reaction can thus be easily shifted in favor of thiol ester formation by exposing the reaction mixture to air.

The high reactivity and selectivity of the choline selenol esters should make these compounds useful for the specific labeling of thiol groups on macromolecules. The thiol ester labels can, in turn, be easily removed by aminolysis. As observed with anisoyl selenol choline, modification of the acyl group does not seem to substantially alter the acylating capability of these selenol esters.

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