In Vitro Biogeneration of Pure Thiosulfinates and Propanethial-*S*-oxide

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A model reaction system was developed for generating pure thiosulfinates and propanethial-*S*-oxide (PTSO) using an isolated alliinase (EC 4.4.1.4) and isolated or synthetic alk(en)yl-L-cysteine sulfoxides (ACSO). Reaction yields ranged from 30 to 60% after 3 h at 21−23 °C, and organosulfur reaction products were extracted into $CHCl_3$ to yield product preparations of controlled composition. A pure thiosulfinate or PTSO was derived from a single ACSO, and a preparation containing a mixture of four thiosulfinate species was derived from reaction mixtures employing binary ACSO substrate systems. Identities of homologous thiosulfinates and PTSO were confirmed by ^{1}H NMR. This approach has the potential to be used as a preparative tool for yielding pure thiosulfinates and PTSO to facilitate the study of chemical and biological properties of this group of compounds or as a means to study the dynamics of organosulfur chemistry in preparations from *Allium* spp.

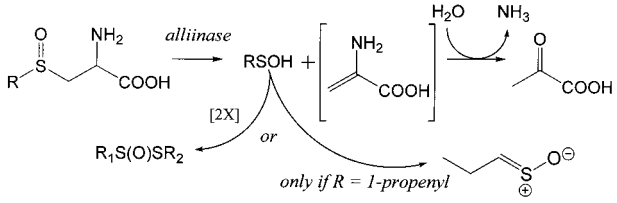
Keywords: *Allium; thiosulfinates; propanethial-S-oxide; biogeneration; alliinase; alk(en)yl-L-cysteine sulfoxides*

INTRODUCTION

Organosulfur components from tissues of *Allium* species have been the focus of decades of study because of their sensory properties (Stoll and Seebeck, 1951) and prospects of possessing therapeutic activities (Kendler, 1987; Lau et al., 1990). In many instances, biological effects have been demonstrated with pastes, distilled oils, or extracts of *Allium* tissues, and these effects have often been correlated with, or attributed to, the organosulfur components intrinsic to the preparation (e.g., Sendl et al., 1992). In some cases, subsequent studies with pure compounds have allowed direct assignments of specific biological activities to specific organosulfur components. However, in other reports, the assignment of biological effects to specific organosulfur compounds remains equivocal. A case in point is the assignment of antioxidant activity in a garlic extract to allicin (Prasad et al., 1995), the principal thiosulfinate in macerated garlic tissues (Lawson et al., 1991; Lawson, 1996; Block et al., 1992), despite the prospect that several other compounds in the extract may be more capable than allicin at inhibiting oxidative processes (Yin and Cheng, 1998).

Great strides have been made in understanding the chemistry of the organosulfur components following the initial enzymic transformation of endogenous *S*-alk(en)-yl-L-cysteine sulfoxides (ACSO) to the corresponding and transient alk(en)yl sulfenic acids (Block, 1992). The intial steps in ACSO transformation yields the corresponding sulfenic acid (RSOH) which can rapidly rearrange (for the 1-propenyl species to form and propanethial-S-oxide, PTSO) and/or condense to form homologous ($R_1 = R_2$) or heterologous ($R_1 \neq R_2$) thiosulfinates (R_1S(O)S R_2) as depicted in Scheme 1.

Scheme 1. Transformation of ACSO to Thiosulfinates and PTSO



Perhaps the most important of the organosulfur components in freshly macerated *Allium* preparations are the thiosulfinates and PTSO, since virtually all other organosulfur compounds arising in processed *Allium* tissues are derived from these compounds. However, understanding the chemistry and fate of these organosulfur compounds in tissue preparations is complicated by the complexity of the tissue milieu and how this impacts further transformation of these compounds.

It became evident to us that having a source of pure thiosulfinates and related organosulfur compounds would allow for an examination of their intrinsic chemical properties and biological activities. Thiosulfinates and related organosulfur compounds have been chemically synthesized or isolated from extracts of *Allium* tissues (Lawson et al., 1991; Block, 1992). However, with few exceptions (Block et al., 1986; Lawson et al., 1991; Morimitsu et al., 1992; Block et al., 1997), there has not been a systematic evaluation of structure−function relationships, in terms of chemical and biological properties, of thiosulfinates and related compounds. Furthermore, the dynamics of the fate of thiosulfinates and PTSO in situ remains enigmatic. With these issues in mind, we sought to develop a model reaction system to facilitate the preparation and study of the full range of thiosulfinate species and PTSO that can be found in various, freshly prepared *Allium* extracts. Our approach was based on using isolated or synthetic ACSO and an

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isolated alliinase (EC 4.4.1.4) preparation in a model reaction systems that simulates the generation of these compounds in situ.

MATERIALS AND METHODS

Materials. All chemicals were obtained from Sigma (St. Louis, MO) or Aldrich (Milwaukee, WI) Chemical Companies unless otherwise noted. All solvents used were chromatography grade. White onion bulbs were purchased from a local retail market.

Preparation of Immobilized C−S Lyase (Alliinase). A crude alliinase preparation was prepared using the preliminary isolation steps described earlier (Thomas and Parkin, 1991) with all steps carried out at 0−4 °C. This procedure involved initial homogenization of onion bulbs in 0.1 M potassium phosphate buffer (pH 7.5) containing 10% glycerol, 0.5% poly(vinylpyrrolidone), 0.5 mM phenylmethylsulfonyl fluoride, 5 mM ethylenediaminetetraacetic acid (EDTA), and 0.05% cysteine, followed by precipitation by 65% saturated with ammonium sulfate at 0−4 °C. After collecting the precipitate, exhaustive dialysis against 0.01 M potassium phosphate buffer (pH 7.5) containing 10% glycerol, 5 mM EDTA, and 0.05% cysteine yielded an alliinase preparation of 1.28 mg protein mL^{-1} (based on Coomassie blue assay; Bradford, 1976) and a specific activity of 2.38 Unit μg^{-1} protein (one Unit is defined as one μmol pryuvate produced min^{-1}).

Immoblization of crude alliinase was carried out according to Thomas and Parkin (1991) by placing equal volumes of enzyme preparation and 4% sodium alginate solution (Protanal 5/60; a gift from Protan Inc., North Hampton, NH) in cellulose dialysis tubing (12 000−14 000 MW cutoff, Spectrum Medical Industries, Los Angeles, CA), followed by immersion in 0.1 M $CaCl_2$ solution to induce gelation. The resulting enzyme-loaded gel was then cut into 5−10 mm thick slices and stored at 4 °C until use. For the purpose of these experiments, the immobilized alliinase was capable of in vitro biogeneration of thiosulfinates after as long as 1 yr in storage at 4 °C.

Preparation of Alk(en)yl-L-cysteine Sulfoxide (ACSO) Substrates. *Synthesis of (±)-*S*-Methyl-*L*-cysteine Sulfoxide (MCSO).* Diasteromeric MCSO was prepared by a modified method of Synge and Wood (1956), by combining 23 mL of *S*-methyl-L-cysteine (0.6 M) with 1.75 mL of 30% hydrogen peroxide. After continuous stirring for 24 h at 21−23 °C, particulate material was removed by filtration, and a white precipitate of MCSO was recovered by adding 250 mL of cold ethanol to the filtrate and holding at 4 °C overnight. Yields were typically 60−80%.

*Synthesis of (±)-*S*-Ethyl-*L*-cysteine Sulfoxide (ECSO).* Synthesis of diasteromeric ECSO was essentially the same as the synthesis of MCSO except that *S*-ethyl-L-cysteine was used as the starting material. Yields were typically 60−75%.

*Synthesis of (±)-*S*-Propyl-*L*-cysteine Sulfoxide (PCSO).* Synthesis of diasteromeric PCSO was similar to the method described by Lancaster and Kelly (1983). L-Cysteine-HCl (4 g) was dissolved in 75 mL of ethanol followed by dropwise addition of 5.6 mL of 20 N NaOH. After 5 min, 4 mL of 1-propyl bromide was added. The mixture was stirred overnight at 21−23 °C and then adjusted to pH 5.5 using acetic acid. The resultant suspension was cooled to 4 °C for 2 h. The *S*-propyl-L-cysteine precipitate was collected by filtration, and the corresponding sulfoxide was formed by oxidizing *S*-propyl-L-cysteine with 30% hydrogen peroxide as described for MCSO synthesis above, with a typical yield of 45−55%.

*Synthesis of (±)-*S*-2-Propenyl-*L*-cysteine Sulfoxide (2-PeCSO).* Synthesis of diasteromeric 2-PeCSO was similar to the synthesis of PCSO described above except that 2-propenyl bromide was substituted for propyl bromide, with yields of about 15−20%.

*Isolation of (+)-*S*-1-Propenyl-*L*-cysteine Sulfoxide (1-PeCSO) from White Onion Bulbs.* (+)-*S*-(1-propenyl)-L-cysteine sulfoxide, the natural form of 1-PeCSO in onion, was obtained by a modified method of Carson et al. (1966) with all procedures done at 21−23 °C except where noted. White onion bulbs (4−5 kg) were cut into quarters and peeled. Batches of 500−600 g tissue segments were placed in about 1 L boiling distilled water for 5 min and then removed to cool. The heated tissue was then homogenized in the heating medium plus another 0.2 volumes of distilled water. The slurry was filtered through cheesecloth, and the filtrate was adjusted to pH 4 with acetic acid before centrifugation (40 min × 13 000*g*) at 4 °C to remove particulate matter.

The resulting supernatant was loaded onto a column (5 × 30 cm) of Dowex 50-X4 (H^+), and the column was eluted with 0.1 M sodium acetate (pH 6.5). Eluting fractions were tested for ninhydrin-positive material by spraying with 0.8% ninhydrin in ethanol after chromatography on Whatman #1 filter paper (butanol−acetic acid−water: 63/10/27, v/v/v as developing solvent). Presumptive 1-PeCSO-containing fractions were identified on the basis of comigration with a 1-PeCSO standard. The 1-PeCSO-containing fractions were pooled, applied to a second column (2.5 × 50 cm) of Dowex 50-X4 (H^+), and eluted with 0.05 N NaOH at 30 mL h^{-1}. Presumptive 1-PeCSO-positive fractions (still in 0.05 N NaOH) were again pooled and passed through a third column (2 × 15 cm) of Dowex 2-X8 (OH^-) to obtain the acidic amino acids, including 1-PeCSO, which are not retained by the column. At this point, ninhydrin-positive fractions showed virtually one band on paper chromatography. Those fractions were finally loaded onto a column (2.5 × 50 cm) of Dowex 50-X4 (H^+), which was eluted with 0.05 N ammonium hydroxide. The 1-PeCSO-containing fractions were pooled, adjusted to pH 6.5 with acetic acid, and lyophilized. Recrystallization of an aqueous solution (1−2 mL) of lyophilized material by dropwise addition of acetone yielded pure 1-PeCSO, with a yield of about 1 g.

The structure of 1-PeCSO was confirmed by ^{1}H NMR (model AM-300 NMR spectrometer, Bruker Instruments, Inc., Billerica, MA) (300 MHz, D_2O): δ 6.56 (*qd*, J = 15, 6.5 Hz, 1H, CCH=CS−), 6.37 (*dd*, J = 15, 1.5 Hz, 1H, CC=CHS−), 3.96 (*dd*, J = 8.5 Hz, 1H, αCH), 3.29 (*ABdd*, J = 14.5 Hz, 1H, $-SCH_a\alpha C-$), 3.10 (*ABdd*, J = 14.8 Hz, 1H, $-SCH_b\alpha C-$), 1.79 (*dd*, J = 6.5, 1.5 Hz, 3H, CH_3C=C−). This ^{1}H NMR spectrum is consistent with the double bond of the 1-PeCSO being in the trans (native) form (Nishimura et al., 1975; Zheng et al., 1988; Mütsch-Eckner et al., 1992).

Model Reaction System. Typically, ACSO (0.05 mmol) was dissolved in 4.0 mL of 100 mM Tris (pH 7.5), and 0.5 g of immobilized alliinase (ground with a mortar and pestle to yield gel fragments of about ≤ 1 mm dimension) was added to initiate the reaction at 21−23 °C. After various periods of incubation, thiosulfinate ($R_1S(O)SR_2$; where R_1/R_2 groups are methyl (Me), ethyl (Et), propyl (Pr), allyl (All), or 1-propenyl (Pren) residues) products were obtained by extracting an aqueous subsample of the reaction mixture into an equal volume of $CHCl_3$ (containing benzyl alcohol as internal standard). The authenticity of the S-containing compounds obtained was confirmed by ^{1}H NMR (300 MHz, $CDCl_3$) as follows (some spectra could be compared to previous reports: Lawson et al., 1991; Naganathan, 1992; Block et al., 1996): *MeS(O)SMe*: δ 3.00 (3H, *s*, $CH_3S(O)-$), 2.69 (3H, *s*, $CH_3SS(O)-$); *EtS(O)SEt:* δ 3.05−3.24 (4H, *m*, $-CH_2S(O)SCH_2-$), 1.48 (3H, *t*, J = 7.5 Hz, $CH_3CS(O)-$), 1.42 (3H, *t*, J = 7.5 Hz, $CH_3CSS(O)-$); *PrS(O)SPr:* δ 3.03−3.22 (4H, *m*, $-CH_2S(O)SCH_2-$), 1.77−1.96 (4H, *m*, $-CH_2CS(O)SCCH_2-$), 1.10 (3H, *t*, J = 7.5 Hz, $CH_3CCS(O)-$), 1.05 (3H, *t*, J = 7.5 Hz, $CH_3CCSS(O)-$); *AllS(O)SAll:* δ 5.87−6.04 (2H, *m*, C=CHC−), 5.20−5.49 (4H, *m*, CH_2=CC), 3.70−3.93 (4H, *m*, C=CCH_2); *PTSO* ($C_2H_5CH=S^+-O^-$): δ 8.18 (1H, *t*, J = 7.8 Hz, −S=CHCC), 2.80 (2H, *p*, J = 7.8 Hz, $-S=CCH_2C$), 1.16 (3H, *t*, J = 7.8 Hz, $-S=CCCH_3$). The lack of a detectable downfield signal at δ 8.86 for PTSO confirmed that the cis (natural) isomer was present (Block et al., 1996).

Quantification of organosulfur analytes was based on the peak area in HPLC chromatography (model 2300 pumps, V^4 detector set at 254 nm, and with peak integration by ChemResearch software; Isco, Lincoln, NE). Normal phase chromatography on a 250 mm × 4.6 mm, Microsorb 5 μm Silica column (Rainin Instrument Co Inc., Woburn, MA) involved gradient elution with 2-propanol:hexane from 2:98 (v/v) held

Table 1. Efficiency of Chloroform Extraction of Thiosulfinates and PTSO from Model Reaction Mixtures

thiosulfinate species	proportion extracted	thiosulfinate species	proportion extracted
EtS(O)SEt	99.7 (4)	AllS(O)SAll	100 (2)
EtS(O)SMe	95.3 (2)	AllS(O)SMe	95.4 (2)
MeS(O)SEt	100 (2)	MeS(O)SAll	98.0 (2)
MeS(O)SMe	87.7 (5)	AllS(O)SEt	103 (2)
PrS(O)SPr	100 (4)	EtS(O)SAll	100 (2)
PrS(O)SMe	99.9 (2)	PrS(O)SEt	97.0 (2)
MeS(O)SPr	102 (2)	EtS(O)SPr	97.5 (2)
PTSO	100 (2)		

for 6 min to 10:90 (v/v) for the next 10 min followed by a 7 min hold, with an elution rate of 1.4 mL min^{-1} (adapted from Block et al., 1992).

Concentration of each of these organosulfur components in a stock solution was determined on the basis of their ^{1}H NMR signal and comparison to the signal of the internal standard (*tert*-butyl alcohol). This allowed the calibration of the HPLC detector response to external standard curves for each analyte. The relationship between concentration and area units (au) using 254 nm detection were as follows: MeS(O)SMe, 68.1 nM au^{-1}; EtS(O)SEt, 87.4 nM au^{-1}; PrS(O)SPr, 85.6 nM au^{-1}; AllS(O)SAll, 80.9 nM au^{-1}; PSTO, 20.9 nM au^{-1}. These values are consistent with the extinction coefficients reported for thiosulfinates in a 2-propanol:hexane mixture (Lawson et al., 1991). Heterologous thiosulfinates were presumptively identified on the basis of the elution patterns observed in Block et al. (1992) and using response factors calculated as the average values of the corresponding homologous thiosulfinates.

The efficiency of $CHCl_3$ extraction of thiosulfinates from reaction mixtures was determined on the basis of back-extraction of standardized thiosulfinate solutions in $CHCl_3$ into an equal volume of water by vortexing for 1 min at 21−23 °C. Results, with a coefficient of variation of about ± 3%, appear in Table 1. For the purposes of this study, corrections for incomplete extraction were applied for MeS(O)SMe ($\times 0.88^{-1}$) and EtS(O)SMe and AllS(O)SMe of ($\times 0.95^{-1}$), whereas all other thiosulfinates and PTSO were considered to be quantitatively extracted within the limits of the ± 3% variance.

The progress of model reactions was also followed by pyruvate formation, based on the coupling assay involving oxidation of NADH to NAD by lactate dehydrogenase (Schwimmer and Weston, 1961). In this case, a 0.3 mL subsample of the reaction mixture was quenched by the addition of 1.8 mL of 2.2% trichloroacetic acid and then centrifuged for 3 min × 13 600*g* to clarify the extract. A 0.5 mL portion of the cleared extract (yielding up to 2 mM pyruvate) was assayed for pyruvate.

For all these studies, at least two duplicate experiments were conducted, and the mean values are reported along with CV for the experimental data set.

RESULTS AND DISCUSSION

Progress of Model Reaction Mixtures. Using reaction conditions that were developed as being "standard", the progress of model reactions for immobilized alliinase and ACSO is shown in Figure 1. Tris buffer at pH 7.5 was used because of the reported optimum of the onion alliinase as being pH 7−8 (Tobkin and Mazelis, 1979; Nock and Mazelis, 1987; Thomas and Parkin, 1991). Under these conditions, pyruvate formation readily took place for all ACSO substrates used, and relative initial rates of reactions were observed in descending order: 1-PeCSO > 2-PeCSO > PCSO > ECSO > MCSO. This order of preference is consistent with the relative reactivity of ACSOs and alliinase predicted on the basis of their respective (and increasing) K_m values for these substrates (Schwimmer et al., 1964; Schwimmer, 1969; Tobkin and Mazelis, 1979). The

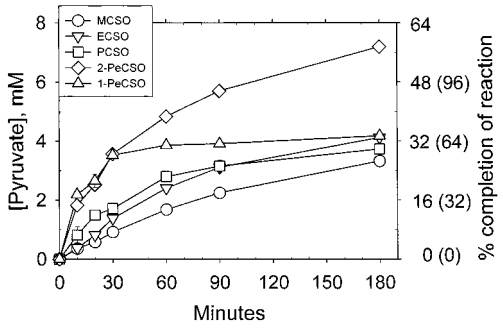


Figure 1. Pyruvate generation in model reaction mixtures. Reaction mixtures contained 0.05 mmol of either methyl, ethyl, propyl, 2-propenyl or 0.025 mmol 1-propenyl cysteine sulfoxide (MCSO, ECSO, PCSO, 2-PeCSO, 1-PeCSO, respectively), 0.5 g of immobilized alliinase in 4.0 mL of Tris (7.5) buffer. Right ordinate denotes % reaction yield (in parentheses for 1-PeCSO) based on the amount of pyruvate formed relative to the initial level of ACSO. CV for duplicate experiments was 6.8%.

fact that the (+) isomer of 1-PeCSO and the (±) diasteromeric mixtures of the other ACSOs were used is of some, but limited, impact. Although the (+) isomer is favored by alliinase over the (−) isomer, when equimolar mixtures of the (±) diastereomers are present, the kinetics of the system reflect those of the faster reacting isomer (Schwimmer et al., 1964).

The data in Figure 1 were also transformed to linear (log−log) plots (not shown) representing the time-dependent fractional conversion of each substrate relative to each other (theory in Deleuze et al., 1987). The slopes of these plots represent the relative values of V_m/K_m (the selectivity constant; Fersht, 1985) for each substrate. Fits for these linear plots ($r^2 \geq 0.94$) yielded respective and relative values for V_m/K_m for the MCSO, ECSO, PCSO, 2-PeCSO, and 1-PeCSO as 1.00:1.59:2.01:4.38:10.7. These value are consistent with respective V_m/K_m values of 1:3:18 for MCSO:PCSO:1-PeCSO (calculated from the kinetic data provided by Freeman and Whenham, 1975).

The reaction progress curves were typical of enzyme reactions except for a rather abrupt decline of activity with 1-PeCSO after 30 min, a point where only about 60% 1-PeCSO was consumed based on the evolution of pyruvate. This phenomenon has been witnessed before for onion alliinase in vitro using 1-PeCSO (Schwimmer, 1969) and in situ toward MCSO and PCSO (Lancaster et al., 1998).

The corresponding progress curves for the formation of thiosulfinates in alliinase/ACSO reaction mixtures (Figure 2; corrected for extraction efficiency) were generally corroborative with the evolution of pyruvate (Figure 1), in that the same order of preferences of ACSO (except for 1-PeCSO, discussed later) reactivity as indicated by pyruvate formation also held for thiosulfinate formation. However, based on the reaction stoichiometry of 2:1 molar ratio of pyruvate:thiosulfinate formed (Scheme 1), less than stoichiometric levels of pyruvate were found for all but one case (Figure 3). Pyruvate levels ranged from 36 to 104% of those expected based on thiosulfinate yields, and the greatest substoichiometric levels of pyruvate were observed early in the reactions for all ACSO substrate systems. Although pyruvate is widely used as an indicator of pungency of *Allium* tissue preparations (Randle and Bussard, 1993), less than stoichiometric yields of pyruvate in alliinase-mediated reactions in situ have been reported previously (Lancaster et al., 1998).

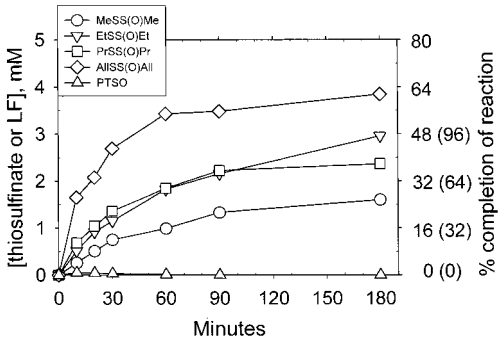


Figure 2. Organosulfur product generation in model reaction mixtures. Legend is the same as for Figure 1, except that products formed were thiosulfinates, RS(O)SR, where R = methyl (Me), ethyl (Et), propyl (Pr), 2-propenyl (All), or propanethial-*S*-oxide (PTSO), from the corresponding ACSO. CV for duplicate experiments was 2.9%.

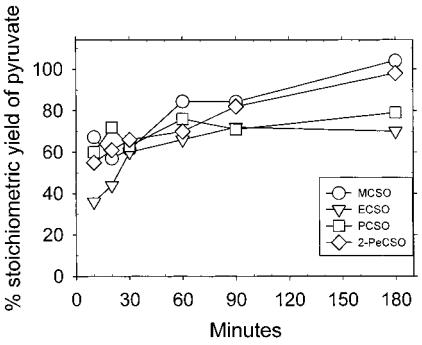


Figure 3. Degree of stoichiometric yield of pyruvate in model reaction mixtures. Yield (%) of pyruvate observed was based on an expected product stoichiometry of 2:1 pyruvate:thiosulfinate for the model reactions. Data and legend are from Figures 1 and 2.

The 70−92% deficit in stoichiometric levels of pyruvate relative to the degree of ACSO transformation previously reported is equivalent to ca. 16−28 mM pyruvate in onion bulb homogenates (calculations are based on data appearing in Table 2 in Lancaster et al., 1998). This unmeasurable pyruvate, or "crypto-pyruvate", was suggested to be underestimated or undetectable because of pyruvate binding to pyridoxal phosphate at the active site (Lancaster et al., 1998). Onion bulb tissue contains about 4 μM pyridoxal phosphate and an upper limit of about 15 g protein kg^{-1} (Fenwick and Hanley, 1985). At a subunit molecular mass of 50 kDa (Nock and Mazelis, 1987) and assuming that alliinase comprises up to about 10% of the protein, a calculated in situ level of onion alliinase of 30 μM indicates that such a mechanism would contribute very little (<0.2%) to the "crypto-pyruvate" phenomenon. Instead, we believe that the majority of substoichiometric levels of pyruvate must be accounted for by other means, possibly in the slow conversion to pyruvate (Schmidt et al., 1996), or instability and/or reactivity of the aminomethacrylic acid intermediate (Scheme 1), or reactivity of pyruvate with other compounds (Chu and Clydesdale 1976; Fulcrand et al., 1998).

The second inconsistency between the trends in Figures 1 and 2 is for reactions with 1-PeCSO as substrate, where much less PTSO was formed than would be predicted based on the levels of pyruvate evolved in this reaction system. Lack of recovery of PTSO may be attributed to volatilization and/or chemical transformation to yield products not detected by our HPLC method over the 2 h reaction period. Subsequent experiments revealed that the fate of PTSO in these reactions systems was dependent on the pH and choice of buffering agent (Figure 4). Reactions taking place at pH 7.5 were faster than those at pH 5.0, using both pyruvate and PTSO formation as indicators, especially considering reaction progress within the first 60 s. This was expected based on the reported pH optimum of onion alliinase to be in the range of 7−8 (Tobkin and Mazelis, 1979; Nock and Mazelis, 1987; Thomas and Parkin, 1991). However, alliinase was sufficiently reactive at pH 5.0 (which is similar to the pH of macerated onion bulb tissue) such that similar extents of ACSO transformation were accomplished at longer incubation times at pH 5.0 relative to shorter incubation times at "optimum pH". Although counterintuitive, it was clear that model reactions at suboptimal pH were more conducive than those at optimal pH for alliinase-mediated generation and recovery of PTSO from 1-PeCSO.

The data in Figure 4 also indicate that the stability/reactivity of PTSO was dependent on pH in the range of pH 5−7, rendering PTSO more sensitive to increasing pH than thiosulfinates (Block, 1992). Also, greater recoveries of PTSO were observed in phosphate-buffered compared to Tris-buffered systems. We attribute this phenomenon to the potential for amine functional groups (Tris) to either react directly with PTSO or serve as a nucleophilic catalyst to facilitate the transformation or decay of PTSO into various derivatives. The relationship that exists between buffering agents and the fate of PTSO in the alliinase/ACSO reaction system is the subject on ongoing study in this laboratory.

Reactions with Binary ACSO Systems. Having established the capability of preparing homologous thiosulfinates from a single ACSO substrate, binary ACSO reaction systems were evaluated for their ability to yield mixtures of homologous and heterologous thiosulfinates (Table 2). The profiles of the four species of thiosulfinates yielded by each binary ACSO system was reflective of the relative rates of activity of the individual ACSO. Both % yield of thiosulfinate and mole fraction of alk(en)yl groups from the faster reacting ACSO comprising the total thiosulfinate pool increased as the binary ACSO system was modified to contain a faster reacting ACSO species or a greater proportion of the faster reacting ACSO species of the pair.

The profile of heterologous thiosulfinates formed in the binary ACSO mixtures generally followed the pattern of $R_1S(O)SR_2 > R_1SS(O)R_2$, where R_1 and R_2 were derived from the slower and faster reacting ACSO, respectively. A sulfenic acid (RSOH)-thiosulfinate alk(en)yl-exchange reaction can account for this behavior where the RSOH reactant is derived principally from the slower reacting ACSO and the homologous thiosulfinate from the faster reacting ACSO (Scheme 2, adapted from Block, 1992; Lawson, 1996). The basis of this mechanism is kinetic, and a greater disparity in reaction rates of different ACSO should lead to a greater magnitude of difference between the heterologous thiosulfinate species. However, we found little correlation ($r^2 = 0.179$, $n = 10$) between the molar ratios of the heterologous and homologous thiosulfinates in the binary ACSO reaction mixtures (data not shown). While the ratios of about 2:1 for the heterologous thiosulfinates in the model reaction mixtures are consistent with what has been found in tissue preparations (Lawson et al., 1991; Block et al., 1992), a theoretical ratio of 3:1 of

Table 2. Thiosulfinate Yields and Profiles in Binary ACSO Reaction Mixtures[c]

	molar ratio of $ACSO_A/ACSO_B$ substrates						
	1:1			2:1			
$ACSO_{A/B}$ substrates	total TS (mM)	TS % yield[a]	TS ratio	total TS (mM)	TS % yield[a]	TS ratio	TS products
MCSO/ETCSO			3.4			1.0	EtS(O)SEt
	2.44	19.5	1.9	2.65	14.1	1.0	EtS(O)SMe
			1.8			0.9	MeS(O)SEt
			1.0			1.0	MeS(O)SMe
MCSO/PCSO			16.4			4.7	PrS(O)SPr
	2.37	19.0	3.1	2.74	14.6	1.7	PrS(O)SMe
			4.8			2.5	MeS(O)SPr
			1.0			1.0	MeS(O)SMe
MCSO/2-PeCSO			23.4			3.6	AllS(O)SAll
	2.91	23.3	2.7	2.89	15.4	1.3	AllS(O)SMe
			5.8			2.4	MeS(O)SAll
			1.0			1.0	MeS(O)SMe
ECSO/PCSO			5.3			1.6	PrS(O)SPr
	2.69	21.5	1.7	3.54	18.9	0.9	PrS(O)SEt
			2.9			1.5	EtS(O)SPr
			1.0			1.0	EtS(O)SEt
ECSO/2-PeCSO			10.4			1.4	AllS(O)SAll
	3.48	27.9	2.1	3.55	18.9	0.9	AllS(O)SEt
			5.0			1.9	EtS(O)SAll
			1.0			1.0	EtS(O)SEt
PCSO/2-PeCSO	4.04	32.3	ND[b]	ND	ND	ND	(individual species were not resolved)

[a] TS yield was calculated based on the amount of ACSO substrate used. [b] Not determined. [c] Reaction conditions were 12.5 mM each ACSO (1:1 molar ratio) or 25.0/12.5 mM ACSO (2:1 molar ratio), 0.8 g of immobilized enzyme in 4 mL of Tris buffer at pH 7.5 and 25 °C for 90 min.

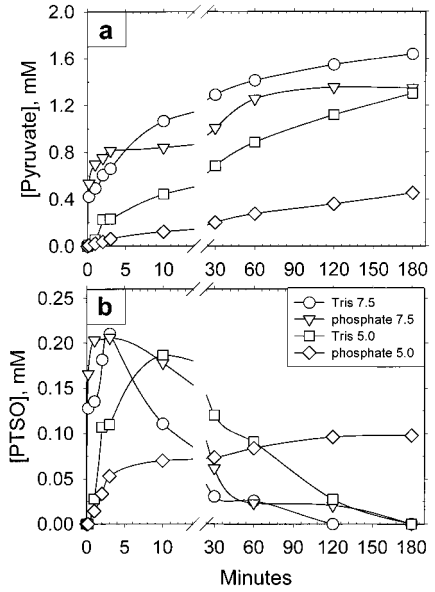
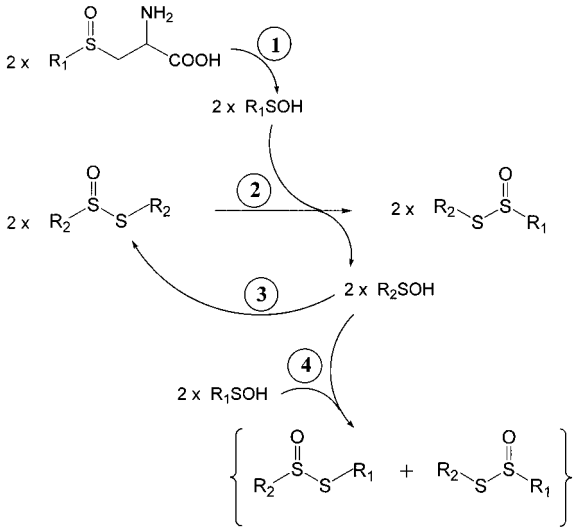


Figure 4. Reaction progress in mixtures containing 1-PeCSO as substrate. (a) Pyruvate formation and (b) PTSO formation as a function of pH, using either Tris or sodium phosphate buffer where reaction conditions are the same as in Figures 1 and 2. CV for duplicate experiments was 15.5% and 9.2% for the determination of pyruvate and PTSO, respectively.

MeS(O)SAll:MeSS(O)All has been predicted in garlic preparations (Lawson, 1996).

An alternative to the kinetic mechanism would be founded on a nonequivalency in reactivity of different RSOH, based on differences in nucleo-/electrophilicity or even steric factors. This may result in a selectivity or enrichment in certain alk(en)yl species linked to the sulfoxide (−S(O)−) moiety during thiosulfinate formation. However, based on the thiosulfinate profile produced in the 2:1 MCSO:ECSO binary mixture, where equimolar levels of R_1 and R_2 groups were evolved as thiosulfinates (Table 2), and the lack of prior evidence for such a mechanism in the literature, we dismissed this alternative mechanism as a possibility.

Scheme 2. Exchange Reactions between Sulfenic Acids and Thiosulfinates



[a] R_1 and R_2 represent alk(en)yl groups originating from ACSO or thiosulfinate, respectively.

Further exploitation of the model reaction system was used to study how the evolution of heterologous thiosulfinates could be manipulated. Reaction systems containing an ACSO and preformed thiosulfinate were initiated by the addition of alliinase under standard conditions, and products were extracted into $CHCl_3$ and analyzed by HPLC. When 13 mM MCSO was reacted in the presence of 6 mM AllS(O)SAll, the ratio of MeS(O)SAll:MeSS(O)All was 26:1 after 2 h reaction. When the conditions were changed to 19 mM MCSO and 7 mM AllS(O)SAll, the ratio of MeS(O)SAll:MeSS(O)All was 14:1 after 2 h reaction. These ratios can be explained using Scheme 2, where a "slow" reacting MCSO (step

(1)) yields MeSOH which is "trapped" by reaction (step (2)) with AllS(O)SAll. This yields MeS(O)SAll and AllSOH (mechanism in Scheme 34 of Block, 1992). The low steady-state levels of MeSOH favor step (3) over step (4) for the fate of residual AllSOH, minimizing the formation of MeSS(O)All (which occurs only by step (4)) in this system. The reaction system with the greater initial MCSO level apparently generated sufficient steady-state MeSOH levels to create flux through step (4), causing the reduced MeS(O)SAll:MeSS(O)All ratio.

When 11 mM 2-PeCSO was reacted with 2.5 mM MeS(O)SMe under standard conditions for 3 h, a molar ratio of MeS(O)SAll:MeSS(O)All of 1:3 was found. Referring to Scheme 2, with 2-PeCSO being considered a "fast" reacting substrate in step (1), only the MeSS(O)All regioisomer is formed in step (2), and AllSOH apparently reached steady-state levels sufficient for making reaction step (4) more favorable than step (3) (in contrast to the MCSO + AllS(O)SAll system discussed above). This was also indicated by the observed product molar ratio of AllS(O)SAll:MeS(O)SAll of 1:1.24 found for this reaction system. Collectively, the results of these experiments are consistent with a kinetic/mass action account of the evolution of regioisomeric thiosulfinates in dynamic systems representative of *Allium* tissue preparations. (Of course, heterologous thiosulfinates can also be formed by the random condensation of simultaneously formed RSOH, where $R_1 \neq R_2$ (Scheme 1)).

CONCLUSIONS

The alliinase-based system for biogeneration of thiosulfinates and PTSO described in this report has several applications. It can be used as a means to prepare sufficient quantities of thiosulfinates (or PTSO), either in pure form or as mixtures of controlled composition, for the systematic evaluation of structure−function relationships. Such functional activities that remain to be explored include biological and selected chemical properties and reactivities. A second potential use would be as a tool to study the dynamics of organosulfur transformation under conditions that simulate ACSO profiles of the various *Allium* species as well as conditions that are used for processing *Allium* tissues into various derivatives. Last, this thiosulfinate- and PTSO-generating system provides a new tool for studying some persistent and poorly understood problems in the processing of *Allium* tissues, such as the nature of bitterness and discoloration in onion and garlic (Fenwick and Hanley, 1990).

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Supporting Information Available: ^{1}H NMR spectra of thiosulfinates, propanethial-*S*-oxide, and 1-propenyl-L-cysteine sulfoxide. This material is available free of charge via the Internet at http://pubs.acs.org.

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