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STUDIES ON CEPHEM SULFONES AS MECHANISM-BASED INACTIVATORS OF HUMAN LEUKOCYTE ELASTASE. IV.[1] REACTIONS ENSUING FROM ENZYMATIC β-LACTAM CLEAVAGE

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Abstract. Interaction of elastase with 7α-methoxycephem sulfones has been investigated by a combination of HPLC and ^{1}H NMR methods. Products arising from enzymatic cleavage have been characterized; observed events were opening of the β-lactam ring, decarboxylation of the resulting acid, opening of the dihydrothiazine ring, and expulsion of the C3' leaving group (if present). In the light of obtained results, putative structures responsible for inhibition and turnover are proposed and discussed.

Human leukocyte elastase (HLE) is the therapeutic target of cephem sulfones as mechanism-based inhibitors, and current efforts in this area aim at selecting a structure with potent, long lasting inhibitory effect coupled to chemical stability.[2] Our approach in this regard is to understand chemical events triggered by reaction with elastase or with chemical nucleophiles and then use this information in the design of new inhibitor structures. In the accompanying paper,[1] the cleavage products of cephem sulfones by nucleophiles have been described and the underlying chemistry has been critically reviewed. Here we present a structural study on products deriving from enzymatic hydrolysis of selected 7α-methoxycephem sulfones (**1a~c**), as obtained upon incubation with either HLE or PPE (porcine pancreatic elastase), and discuss (in light of the previously accumulated knowledge) possible paths leading to their generation, with particular emphasis on the nature of the covalent enzyme-inhibitor complexes. We anticipated a fruitful harvest from a comparison of results obtained with **1a** and **1b**, **1c**, as the presence of a leaving group at C3' position is a recognized structural element associated with potent and long lasting inhibition.[3]

The cleavage products of cephem sulfones were unstable in acidic solution and did not withstand most usual manipulations. We succeded in their characterization by a combination of HPLC analysis using ion-pairing conditions[4] and ^{1}H NMR spectroscopy of the incubation mixtures in H_2O solution.[5] Some structural information was also obtained from the UV spectra of HPLC peaks. Compound **1a** behaved as a poor substrate rather than as an inhibitor, since it was slowly but thoroughly converted into cleavage products upon incubation with HLE or PPE. During the first hour of incubation at 37°C with PPE, a transient intermediate (**4**) was identified[6] by NMR, which rapidly decomposed into **5** and **6** (see Figure 1 and Scheme 1). The Z-configuration of **4** was confirmed by the coupling constant (J = 4.3 Hz). The structure of **5** was unambiguously determined by comparison with an authentic sample of methoxyacetaldehyde hydrate.[7] Product **6** (λ_{max} = 290 nm) was sufficiently stable to be detected by HPLC; its structural assignment was based on NMR data[6] and on

comparison with UV absorption properties of related compounds.[8] Significantly, by repeating the NMR investigation in D_2O, deuterium was not incorporated into the olefinic skeleton of **4**. Since the sulfinate salts characterized in chemical cleavage studies were prone to loss of SO_2,[1] the possible presence of bisulfite in the enzymatic cleavage solution of **1a** was investigated by the use of Ellman's reagent, 5,5'-dithiobis-2-nitrobenzoic acid.[9,10] Quantitative HPLC analysis after calibration with sodium bisulfite revealed formation of 1.2 ± 0.2 moles of SO_2 adduct (Bunte salt: RS-SO_3^-)[11] and, quite unexpectedly, 3.3 ± 0.2 moles of free thiol (RSH) per mole of **1a** processed.[12] Structural analysis with NMR monitoring[6] revealed transient formation of imine (**7**), rapidly decomposing into methoxyacetaldehyde (**5**) and poorly identified compounds. These results were interpreted by assuming that the Bunte salt did not arise from free bisulfite, but from reaction with a sulfinate species (possibly **4**), and that a redox process involving a second equivalent of Ellman's disulfide followed. Reaction between disulfides and sulfinic acids[13] (RS-SR + R'SO_2H ⇌ RSH + R'SO_2-SR) is reversible and shifted to the left[14] (it constitutes a synthetic methodology for the preparation of dissymmetric disulfides). However, equilibrium might be displaced when the reactive species is enamine (**4**), owing to irreversible expulsion of the RS-SO_2^- group to generate the conjugated imine (**7**). In turn, the RS-SO_2^- fragment in the presence of Ellman's reagent is expected to undergo rapid oxidation to the corresponding Bunte salt with release of two additional equivalents of the free thiol: RS-SO_2^- + RS-SR + H_2O → RS-SO_3^- + 2RSH.

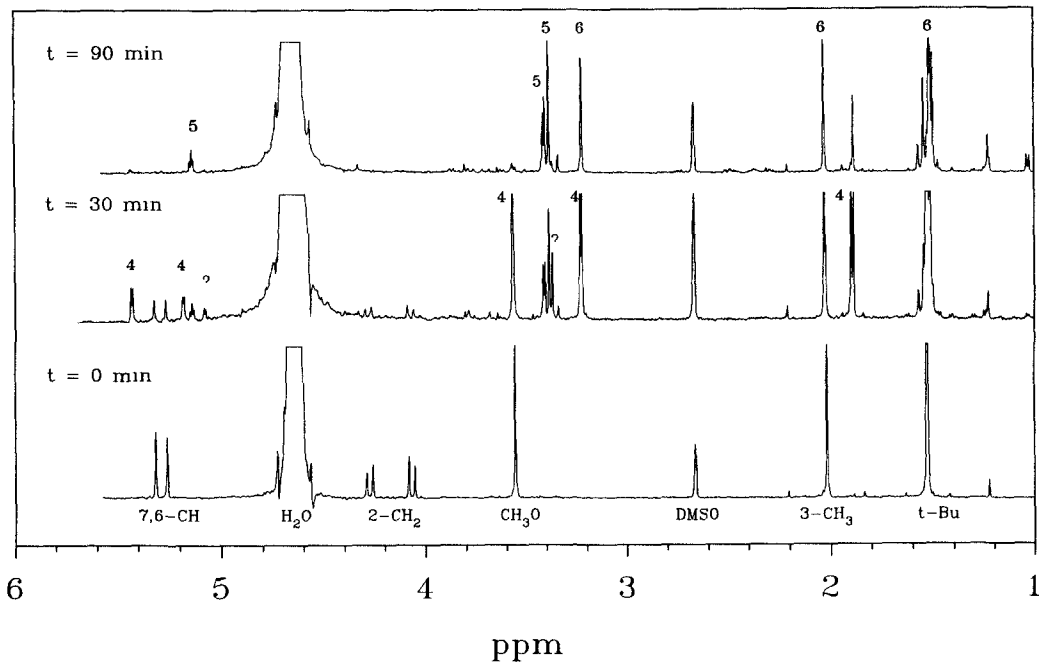
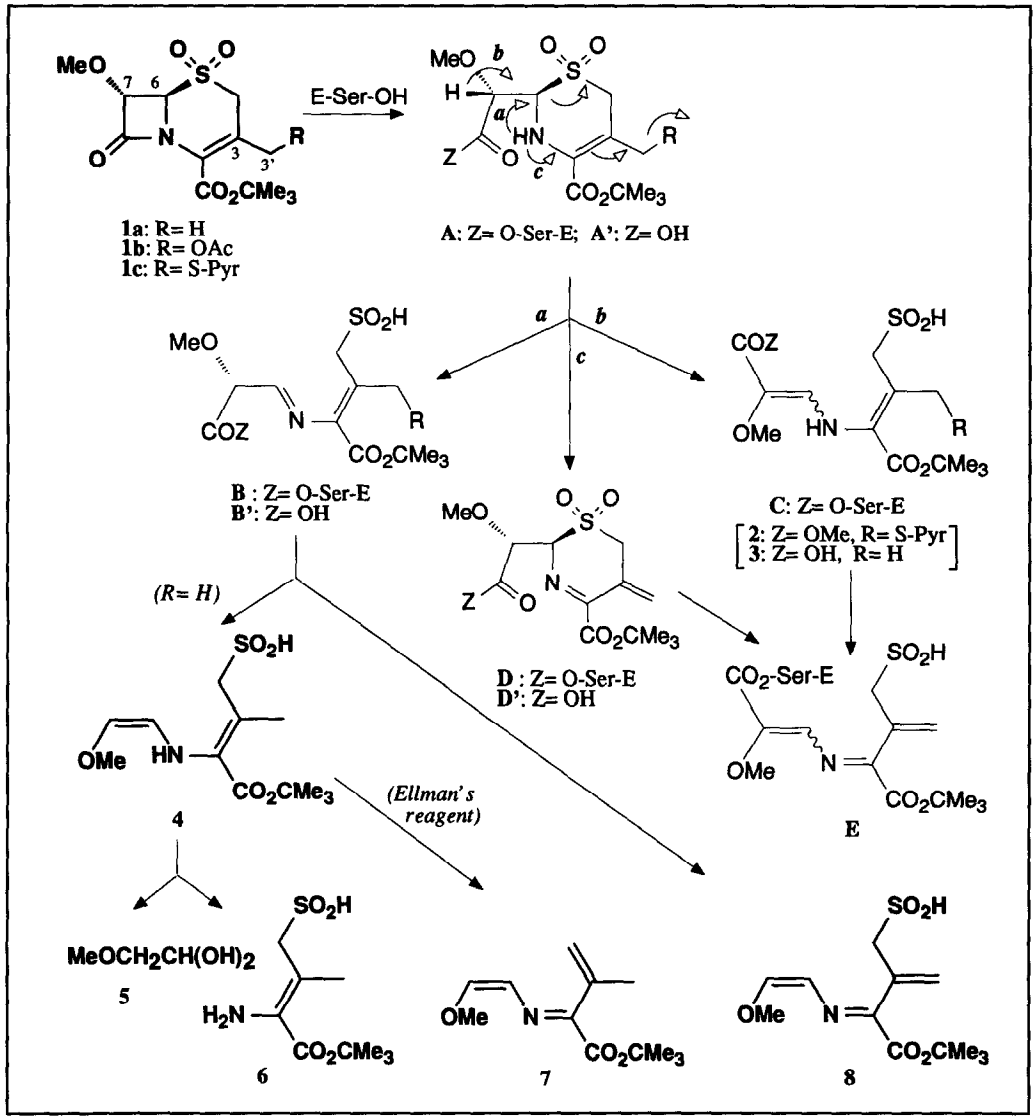


Figure 1. ^{1}H NMR spectra (600 MHz) of incubation mixtures (pH 7.4, 37 °C) containing cephem sulfone (**1a**) and PPE (ca. 1 mg each) in 2 mL of H_2O/DMSO-d_6 (10:1). Peak labels give attribution of resonances to the enzymatic hydrolysis products (Scheme 1). Peaks of an unidentified transient species are labeled with a question mark. The starred peak indicates a resonance due to acetate ion in the PPE preparation.

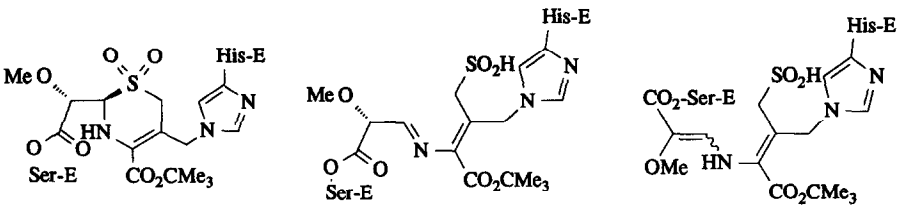


Scheme 1. Proposed chemical mechanisms for the inhibition and hydrolytic turnover of HLE by cephem sulfones. Characterized structures are drawn boldface. Compounds **2** and **3**, representatives of structure **C**, were obtained by chemical cleavage. S-Pyr = 4-pyridylthio.

Compound **1b**, a 3'-activated cephem sulfone, very efficiently inhibited HLE: only 2.7 equivalents were required to completely inactivate one equivalent of the enzyme. This efficience, however, resulted in low yields of cleavage products in solution and, disappointingly, structural studies were frustrating. More rewarding was the investigation of elastase cleavage products of **1c**, which was turned over by HLE at least 30 times before

durable inhibition. HPLC analysis of incubation mixtures of **1c** and HLE or PPE revealed formation of one major product (**8**) with a broad absorption band centered at 300 nm. The structure of **8**, as elucidated by NMR,[6] revealed expulsion of the 4-pyridylthio group and decarboxylation with formation of the same *Z*-olefinic moiety as compound **4**.

Scheme 1, showing possible pathways by which the compounds characterized in the present study were generated by enzymatic turnover, can also be used to discuss possible modes of inhibition according to the available evidence. The β-lactam ring of sulfones (**1a~c**) is initially cleaved by the active site Ser-195 hydroxyl[3,15,16] to generate the transient acyl-enzyme (**A**). Next, two distinct sets of possibilities exist according to whether the original molecule possesses a leaving group at C3'. If no leaving group is present, deacylation to regenerate the free enzyme may occur on the initial acyl-enzyme (**A**→ **A'**) or after dihydrothiazine opening according to path ***a*** (**A**→ **B**→ **B'**). Path ***b*** does not take place, since the β-aminoacrylic acid (**3**) anticipated from deacylation of **C** was observed in alkaline hydrolysis[1] but not in the enzymatic reaction. Subsequently, decarboxylation[17] of either **A'** or **B'** yields the first observed product, bis-enamine (**4**). In turn this compound, not stabilized as acrylate analogs (**2**, **3**) by the electron-withdrawing COZ group, undergoes hydrolytic fragmentation to methoxyacetaldehyde (**5**) and the sulfinic moiety (**6**). This sequence of events was monitored on the 3-methylcephem sulfone (**1a**), which behaved as a substrate rather than as an inhibitor. However, other cephem sulfones devoid of leaving groups at C3' can produce variable degrees of temporary inhibition.[3,18] That might be due to a delay in the deacylation of the initial complex, which might promote generation of **C** (either from **A** via path ***b***, or from **B** by tautomerism), by analogy with chemical cleavages observed in protic solvents (e.g., **1a**→ **3**, **1c**→ **2**).[1] The vinylogous urethane structure (**C**) is commonly associated with a reduced rate of enzyme turnover (temporary inhibition).[19] If a leaving group is present at C3' in the original cephem sulfone, possibilities exist for partitioning between prolonged inhibition and rapid turnover. The product of enzymatic turnover of the 3'-(4-pyridylthio)cephem (**1c**) was the decarboxylated enamine-imine (**8**), lacking the original thiopyridine group. On the other hand, the methanol-cleaved species was the bis-enamine (**2**), still retaining that group.[1] The accompanying questions are on the origin of **8**,[20] the nature of the inhibited species, and the common intermediate partitioning between turnover and inhibition. Expulsion of the leaving group is also proper of the inhibition branch, as shown on the Merck compounds L-658758 and L-659286,[16] but must occur after partitioning, since **1b** and **1c**, differing only for the C3' group, highly differed for the turnover number. Thus, the partitioning acyl-enzyme can be **A** or **B**, not **D** (which is common to **1b** and **1c**) or **C** (which is not a plausible precursor for decarboxylation to **8**). The present study does not provide direct evidence on the structure of the inhibited acyl-enzyme; this can only be obtained after isolation and spectroscopic or crystallographic analysis. The structure of the acyl-enzyme resulting from porcine pancreatic elastase and the 7α-chloro analog of **1b**, solved at 1.84 Å resolution,[21] showed elimination of the elements of hydrochloric acid across C6-C7 and covalent binding of the imidazole ring of His-57 to the ex-C3' carbon atom (double-hit). Subsequent studies revealed that the C7 methoxy group is retained in the complex between HLE and 7α-methoxycephem sulfones, which led to the proposal of the alternative double-hit species (**F**) and (**G**).[3,16]



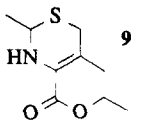
Our observations on the chemical lability of dihydrothiazine sulfones make both structures unlikely, unless unpredictable extra-stabilizations are offered by the active site microenvironment. In fact, **F** is prone to breakage of the C6-S bond (cephem numbering), and **G** to imine-enamine tautomerism; both would rapidly yield **H**. Thus, the latter is a more plausible representation of permanent enzymatic inhibition by a double-hit process in 7α-methoxycephem sulfones. Yet, a word of caution is suggested by the fact that the enzyme-inhibitor complexes in this class of compounds undergo slow spontaneous reactivation.[16,18] Proposal of species (**E**) for this type of durable but reversible inhibition is attractive. First, **E** is chemically congruent, resembling the product of enzymatic turnover (**8**). Second, it is a vinylogous urethane (with extended conjugation). Third, it lies on a reasonable pathway leading to a double-hit adduct (**H**). The acyl-enzyme (**E**) has possible precursors in **B**, **C** or **D**. Whatever the precursor, it is interesting to remark that the enzymatic process (either resulting in inhibition or turnover) entails expulsion of the C3' leaving group, while methanolysis (and β-lactam cleavage by other chemical nucleophiles)[1] does not. The possibility that the active site His-57 provides anchimeric assistance to expulsion of the C3' moiety is one of the fascinating aspects of this chemistry.

In conclusion, the products from enzymatic turnover of a cephem sulfone with no leaving group at C3' (**1a**) and of its C3'-thiopyridyl analogue (**1c**) have been characterized. Combined evidence from chemical and enzymatic reactions ruled out the stability of general structures proposed as representative of the acyl-enzyme complex (**F**, **G**), and allowed the formulation of new ones (**E**, **H**). Monitoring enzymatic turnover reactions and model chemical reactions in synergism is a valuable approach for elucidating the mechanism of action of β-lactam inhibitors.

References and Notes

1. Part III of this series: Alpegiani, M.; Bissolino, P.; Borghi, D.; Rizzo, V.; Perrone, E. *Biomed. Chem. Lett.*, preceding paper in this issue.
2. Finke, P.E.; Shah, S.K.; Ashe, B.M.; Ball, R.G.; Blacklock, T.J.; Bonney, R.J.; Brause, K.A.; Chandler, G.O.; Cotton, M.; Davies, P.; Dellea, P.S.; Dorn, C.P.; Fletcher, D.S.; O'Grady, L.A.; Hagmann, W.K.; Hand, K.M.; Knight, W.B.; Maycock, A.L.; Mumford, R.A.; Osinga, D.G.; Sohar, P.; Thompson, K.R.; Weston, H.; Doherty, J.B. *J. Med. Chem.* **1992**, *35*, 3731.
3. Shah, S.K.; Brause, K.A.; Chandler, G.O.; Finke, P.E.; Ashe, B.M.; Weston, H.; Knight, W.B.; Maycock A.L.; Doherty, J.B. *J. Med. Chem.* **1990**, *33*, 2529.
4. Chromatographic separation was achieved under ion pairing conditions with a Whatman partisphere C18 column (5 μm, 125x4.6 mm), eluted at 1.5 mL/min flow with an appropriate water/acetonitrile gradient in the presence of constant 0.04 M phosphate, pH 6.2, and 1.8 mM tetrabutylammonium bisulfate.

5. In order to detect signals of exchangeable protons, ^{1}H NMR spectra (600 MHz, Varian Unity 600) were recorded under water suppression conditions with H_2O solutions buffered with 50 mM phosphate, pH 7.4, and containing 10% DMSO-d_6 as a solubilizing vehicle for the cephem esters and for NMR lock signal. Solutions in D_2O/DMSO-d_6 were used to verify deuterium uptake at specific positions. PPE was used in these studies, because of its higher stability in the presence of organic co-solvents.
6. ^{1}H NMR assignment of characteristic signals of identified cleavage products:
4: δ 1.50 (9H, s, t-Bu), 1.89 (3H, s, CH_3-C=C), 3.22 (2H, s, $CH_2SO_2^-$), 3.56 (3H, s, CH_3O), 5.17 (1H, d, *J* = 4.3 Hz, C=CH-N), 5.41 (1H, d, *J*= 4.3 Hz, O-CH=C).
5: δ 3.37 (3H, s, CH_3O), 3.40 (2H, d, *J* = 6.0 Hz, CH_2), 5.13 (1H, t, *J* = 6.0 Hz, CH).
6: δ 1.50 (9H, s, t-Bu), 2.01 (3H, s, CH_3-C=C), 3.21 (2H, s, $CH_2SO_2^-$).
7: δ 1.47 (9H, s, t-Bu), 1.80 (3H, s, CH_3-C=C), 3.79 (3H, s, CH_3O), 5.42 and 5.73 (2H, 2s, C=CH_2), 5.95 (1H, d, *J* = 4.6 Hz, C=CH-N), 6.23 (1H, d, *J* = 4.6 Hz, O-CH=C).
8: δ 1.51 (9H, s, t-Bu), 3.39 (2H, s, $CH_2SO_2^-$), 3.80 (3H, s, CH_3O), 5.58 and 5.83 (2H, 2s, C=CH_2), 5.96 (1H, d, *J* = 4.6 Hz, C=CH-N), 6.23 (1H, d, *J* = 4.6 Hz, O-CH=C).
7. A sample of methoxyacetaldehyde was obtained from 2-methoxyethanol by oxidation with pyridinium dichromate (CH_2Cl_2, 1 h).
8. The absorption maximum of dehydrovaline methyl ester (λ_{max} = 265 nm: Cheney, J.; Moores, C.J.; Raleigh, J.A.; Scott, A.I.; Young, D.W. *J. Chem. Soc. Perkin Trans. 1* **1974**, 986) is apparently in contrast with the structural assignment of **6**, however γ-substitution with sulfur may induce a red shift of about 20 nm (cf. **9**, λ_{max} = 285 nm: Demarco, P.V.; Nagarajan, R. *Cephalosporins and penicillins. Chemistry and Biology*; Flynn E.H., Ed., Academic Press: New York, 1972, 363).



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9. Monitoring the HLE catalysed cleavage of **1a** with the method of West & Gaeke ascertained production of SO_2 up to 0.96 ± 0.05 moles per mole of cephem sulfone. However, this procedure involves strongly acidic conditions, which may promote hydrolytic decomposition of the original enzyme cleavage products. West, P.W.; Gaeke, G.C. *Anal. Chem.* **1956**, *28*, 1816.
10. Humphrey, R.E.; Ward, M.H.; Hinze, W. *Anal. Chem.* **1970**, *42*, 698.
11. Distler, H. *Angew. Chem. Internat. Ed.* **1967**, *6*, 544.
12. Spectrophotometric determination of 5-thio-2-nitrobenzoate (ε_{412} = 13,600 $M^{-1}cm^{-1}$: Silverstein, R.M. *Anal. Biochem.* **1975**, *63*, 281) consistently detects 2.86 ± 0.09 moles of liberated free thiol per mole of **1a** after long lasting incubation with HLE.
13. Parson, T.F.; Boockman, J.D.; Pearson, D.E.; Field, L. *J. Org. Chem.* **1965**, *30*, 1923.
14. In fact, reaction of sodium phenylsulfinate with Ellman's reagent was unproductive.
15. Doherty, J.B.; Ashe, B.M.; Argenbright, L.W.; Barker, P.L.; Bonney, R.J.; Chandler, G.O.; Dahlgren, M.E.; Dorn, C.P.; Finke, P.E.; Firestone, R.A.; Fletcher, D.; Hagmann, W.K.; Mumford, R.A.; O'Grady, L.; Maycock, A.M.; Pisano, J.; Shah, S.; Thompson, K.R.; Zimmerman, M. *Nature* **1986**, *322*, 192.
16. Knight, W.B.; Maycock, A.L.; Green, B.G.; Ashe, B.M.; Gale, P.; Weston, H.; Finke, P.E.; Hagmann, W.K.; Shah, S.K.; Doherty, J.B. *Biochemistry* **1992**, *31*, 4980.
17. Likely, decarboxylation occurs on **B'**; decarboxylation of β-iminoacrylates to produce enamines is documented: Knight, W.B.; Green, B.G.; Chabin, R.M.; Gale, P.; Maycock, A.L.; Weston, H.; Kuo, D.W.; Westler, W.M.; Dorn, C.P.; Finke, P.E.; Hagmann, W.K.; Hale, J.J.; Liesch, J.; MacCross, M.; Navia, M.A.; Shah, S.K.; Underwood, D.; Doherty, J.B. *Biochemistry* **1992**, *31*, 8160. Lack of solvent deuterium incorporation at the ex-C7 position indicates that the enamine-imine tautomerism (**B** ⇌ **C**) does not take place in neutral solutions.
18. V. Rizzo, unpublished results.
19. Pratt, R.F. The Chemistry of β-Lactams; Page, M.I., Ed.; Blackie Academic & Professional: London, **1992**; pp 229-271.
20. Decarboxylation of **B'** (R = S-Pyr) is expected to occur readily and provide **8**. Alternatively, 1,4-elimination from the original dihydrothiazine (**A** or **A'**), a process well documented at the sulfide oxidation level,[19] would provide **D'** and thence **8** by decarboxylation and ring opening.
21. Navia, M.A.; Springer, J.P.; Lin, T.-Y.; Williams, H.; Firestone, R.A.; Pisano, J.M.; Doherty, J.B.; Finke, P.E.; Hogsteen, K. *Nature* **1987**, *327*, 79.