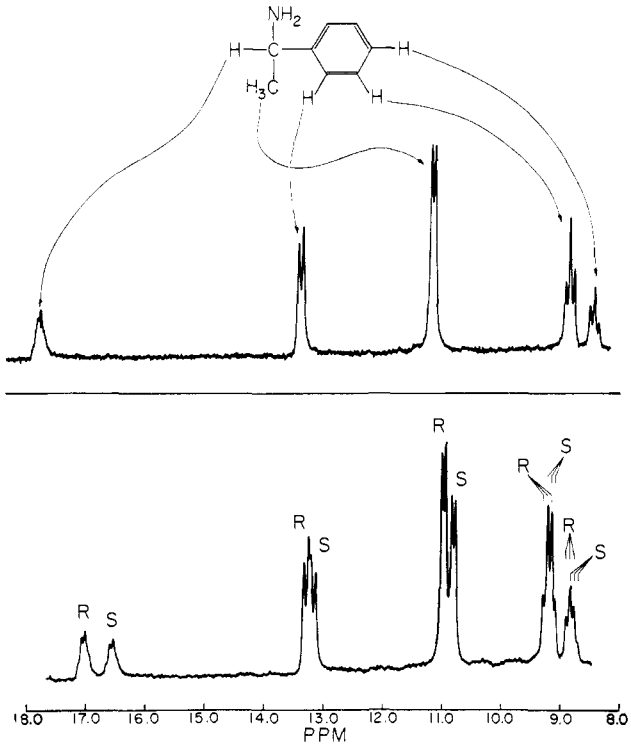
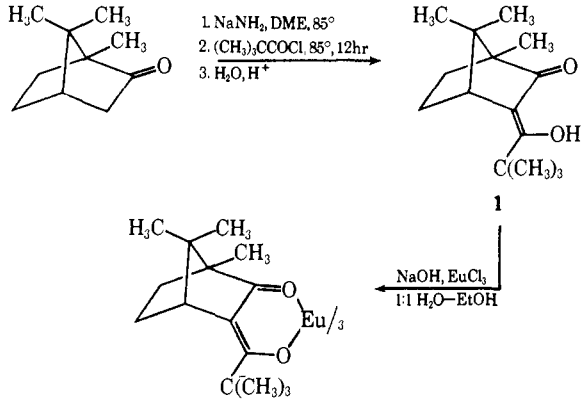


Figure 1. Spectra of solutions prepared from (*S*)-α-phenylethylamine (10 μl) (upper), and a mixture of (*R*)- and (*S*)-α-phenylethylamine (7 and 5 μl, respectively), in 0.3 ml of a carbon tetrachloride solution of **2** (~0.15 *M*). The chemical-shift scale applies only to the spectrum of the mixture; that of the pure *S* enantiometer was displaced slightly to lower field due to differences in concentrations of the samples.

ropium(III) ion and a rapidly exchanging mixture of coordinated and free amine.[6] More noteworthy in



1

2

these spectra are the frequency *differences* in the resonance of corresponding protons of (*R*)- and (*S*)-**3**, ranging from ~0.5 ppm for the C*H*NH$_2$ proton to ~0.07 ppm for the para hydrogen of the aromatic ring. These separations depend on the concentration of **2**: the difference between the C*H*NH$_2$ resonances of ~0.3 *M* solutions of **3** is too small to be observable when [**2**] = 0.015 *M*, reaches a maximum of 0.55 ppm for [**2**] = 0.14 *M*, and decreases to 0.4 ppm at [**2**] = 0.50 *M*.

(6) The potential of lanthanide ions as nmr shift reagents in organic structural analysis was first demonstrated by C. C. Hinckley, *J. Amer. Chem. Soc.*, **91**, 5160 (1969); *J. Org. Chem.*, **35**, 2834 (1970). See also J. K. M. Sanders and D. H. Williams, *Chem. Commun.*, 422 (1970); J. Briggs, *et al.*, *ibid.*, 749 (1970).

These frequency shifts probably reflect differences in the stability constants for the diasteriomeric complexes formed between (*R*)-**3** or (*S*)-**3** and **2**, since an alternative explanation for their origin, involving unequal magnitudes for the pseudocontact shifts within diastereomeric amine–europium complexes of *equal* stability, cannot easily be used to rationalize the fact that *all* of the protons of (*R*)-**3** resonate at lower field than their counterparts in (*S*)-**3**. The frequency shifts between the C*H*NH$_2$ resonances of (*R*)- and (*S*)-**3** in the presence of the praseodymium analog of **2** reached 0.67 ppm; however, the resolution obtained in the presence of this reagent was appreciably lower than that in solutions of **2**.

Other enantiomeric amines exhibit useful spectral differences in the presence of **2**. Thus, for example, the C*H*NH$_2$ resonances of (*R*)- and (*S*)-amphetamine were separated by 0.7 ppm when their pseudocontact shifts reached ~17 ppm from TMS, and the C*H*$_2$-CHNH$_2$ resonances of (*R*)- and (*S*)-2-aminobutane were separated by 1.4 ppm at shifts of ~12 ppm from TMS. On the other hand, shifts between corresponding resonances of less strongly basic enantiomeric substances were generally too small to be useful, although these resonances were still subject to appreciable pseudocontact shifts. Thus, while the C*H*OH resonances of (*R*)- and (*S*)-2,2,6,6-tetramethyl-4-heptyn-3-ol differed by 0.1 ppm at shifts of ~6.9 ppm from TMS ([**2**] ~ 0.3 *M*), distinguishable shift differences were not observed for resonances of 2-octanol, α-phenylethanol, cyclohexylmethylcarbinol, or benzylmethyl sulfoxide.

The use of **2** to determine enantiomeric purities of amines is complementary to techniques employing optically active solvents. The potential of **2** rests in the large shifts observed between resonances of enantiomers in its presence, and in the spectral simplification common to shift reagents;[6] however, these advantages are gained at the expense of a loss in spectral resolution.

Further study of the application of reagents resembling **2** to the direct determination of enantiomeric purity will be described later.

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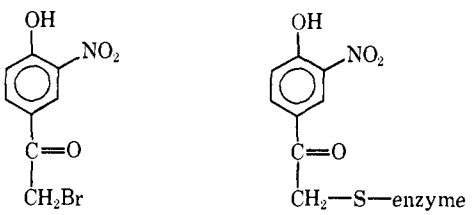
Reaction of the Active Site of Papain with a "Reporter" Group Labeled Phenacyl Halide

Sir:

Despite the recent advances made in the investigation of the cysteine proteinases, the chemistry of the most thoroughly studied enzyme in this group, papain, remains far less developed than that of chymotrypsin, the best understood of the serine proteinases.[1,2] Although the primary amino acid sequence[3] and the three-dimensional structure[4] of papain are now known and

(1) M. L. Bender and F. J. Kézdy, *Annu. Rev. Biochem.*, **34**, 49 (1965).
(2) D. M. Blow and T. A. Steitz, *ibid.*, **39**, 716 (1970).
(3) R. E. J. Mitchel, I. M. Chaiken, and E. L. Smith, *J. Biol. Chem.*, **245**, 3485 (1970).
(4) J. Drenth, J. N. Jansonius, R. Koekoek, H. M. Swen, and B. G. Wolthers, *Nature (London)*, **218**, 929 (1968).

chemical cross-linking experiments have shown that Cys-25 and His-159 are in close proximity to each other at the active site,[5] the pathway of catalysis involving these residues is still the subject of some controversy.[6,7] Furthermore, even though an active-site titration method based on the use of the reactive nitrophenyl ester *p*-nitrophenyl *N*-benzyloxycarbonyl-L-tyrosinate has been described,[8] this method is much less convenient than those commonly used for the active-site titration of α-chymotrypsin.[9,10] This is singularly unfortunate because the development of a rapid and accurate active-site titration procedure for papain is essential if the chemistry of this enzyme is to be placed on a completely quantitative basis. In the present communication we report our discovery that the phenacyl halide α-bromo-4-hydroxy-3-nitroacetophenone[11] (I) reacts rapidly with the active site of papain to produce an inactive modified enzyme, II, containing an *o*-nitrophenol "reporter"[12] group covalently bound to the very center of the active site.



I II

Titration of the active site of papain[13] with I was accomplished by two different experimental approaches. One approach involved direct spectrophotometric titration utilizing differences in the ultraviolet absorption spectra of the chromophoric group of the reagent I and the bound species II. Solutions of papain in 0.067 *M* phosphate buffer at pH 7.0 were very conveniently titrated by this procedure. Observations were made at 322.8 mμ on a Cary 15 spectrophotometer and the increase seen in the extinction coefficient at this wavelength due to the reaction of I with papain to produce II, $\Delta\epsilon = \epsilon_{II} - (\epsilon_I + \epsilon_{papain})$, was 6850.

In the other approach to the titration of papain's active site the inhibition of enzymatic activity due to the reaction of I to give II was measured by rate assays employing *p*-nitrophenyl *N*-benzyloxycarbonylglycinate[14] (III) and ethyl *N*-benzoylargininate[8,15] (IV) as the substrates. When III was used as the substrate, assays were performed at 400 mμ in phosphate buffer at pH 6.8. Assays with IV were carried out at 253 mμ in acetate buffer at pH 5.2. As seen in Figure 1, the hydrolyses of these substrates in the appropriate buffers catalyzed by papain obtained from the same stock solution were inhibited equivalently by the addition of equal amounts of I. Evidence that the sulfhydryl group at the active site of papain is the function reacting with I to give the inhibited enzyme was provided by our observation that the extent of inhibition was paralleled by the loss of the sulfhydryl groups as measured by reaction with 5,5′-dithiobis-(2-nitrobenzoic acid).[16] Furthermore, excellent correspondence was found between the results of direct spectrophotometric titration and the rate assay measurements, indicating that the concentration of the same active species is being measured by the two experimental approaches.

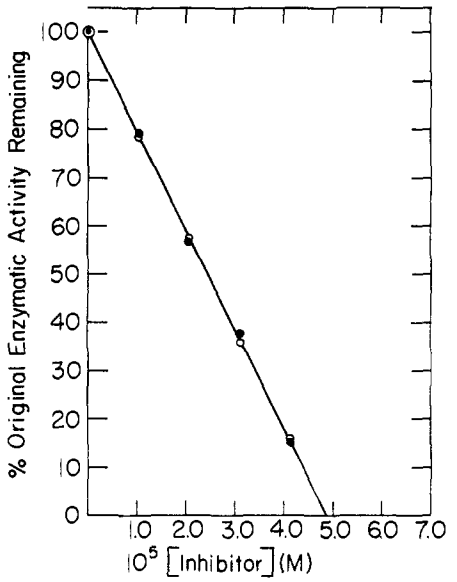


Figure 1. The activity of papain inhibited by varying amounts of 4-hydroxy-3-nitro-α-bromoacetophenone is plotted relative to that of uninhibited enzyme. The open circles represent points which were obtained at pH 6.8 using *p*-nitrophenyl *N*-benzyloxycarbonylglycinate[14] as the substrate. The points represented by closed circles were obtained at pH 5.2 using ethyl *N*-benzoyl-L-argininate as the substrate.[15] The stock solution of papain which was employed was prepared by activation of mercury-papain purified by the method of Blumberg, *et al.*[13]

Finally, titration of the phenolic group in the reporter group labeled species II gave a simple sigmoidal curve, and the pK_a value measured was 5.0. The pK_a value found for the ionization of the phenolic group in I was 4.7.

In summary, the phenacyl halide I has been employed to titrate the active site of papain by two experimental approaches. This facilitates greatly the quantitative investigation of papain-catalyzed reactions. Also, our observations show that although the reporter group in II is located at the active site of papain, the ionization of the *o*-nitrophenol group in this species is not perturbed by any ionizing groups in the enzyme. This is in marked contrast to the results of experiments with another enzyme, α-chymotrypsin,[17] where reporter groups covalently linked to the center of the enzyme

(5) S. S. Husain and G. Lowe, *Biochem. J.*, **108**, 861 (1968).
(6) S. S. Husain and G. Lowe, *ibid.*, **108**, 855 (1968); G. Lowe, *Phil. Trans. Roy. Soc. London, Ser. B*, **257**, 237 (1970).
(7) L. A. Ae. Sluyterman and B. G. Wolthers, *Proc. Konl. Ned. Akad. Wetensch., Ser. B*, **72**, 14 (1969); J. Drenth, J. N. Jansonius, R. Koekoek, L. A. Ae. Sluyterman, and B. G. Wolthers, *Phil. Trans. Roy. Soc. London, Ser. B*, **257**, 231 (1970).
(8) M. L. Bender, M. L. Begué-Canton, R. L. Blakely, L. J. Brubacher, J. Feder, C. R. Gunter, F. J. Kézdy, J. V. Killheffer, Jr., T. H. Marshall, C. G. Miller, R. W. Roeske, and J. K. Stoops, *J. Amer. Chem. Soc.*, **88**, 5890 (1966).
(9) G. R. Schonbaum, B. Zerner, and M. L. Bender, *J. Biol. Chem.*, **236**, 2930 (1961).
(10) J. H. Heidema and E. T. Kaiser, *Chem. Commun.*, 300 (1968); F. J. Kézdy and E. T. Kaiser, *Methods Enzymol.*, in press.
(11) G. Sipos and R. Szabo, *Acta Phys. Chem.*, 7, 136 (1961).
(12) M. B. Hille and D. E. Koshland, Jr., *J. Amer. Chem. Soc.*, **89**, 5945 (1967).
(13) Worthington papain, Lots No. OEA and 9KA, were purified by affinity chromatography. We thank Dr. I. Schechter for generously providing us with a preprint of an article describing this method (S. Blumberg, I. Schechter, and A. Berger, *Eur. J. Biochem.*, **15**, 97 (1970)).
(14) J. F. Kirsch and M. Igelstrom, *Biochemistry*, **5**, 783 (1966).
(15) J. R. Whitaker and M. L. Bender, *J. Amer. Chem. Soc.*, **87**, 2728 (1965).
(16) G. L. Ellman, *Arch. Biochem. Biophys.*, **82**, 70 (1959).
(17) E. T. Kaiser, *Accounts Chem. Res.*, **3**, 145 (1970).

active site were used to detect the ionization of a nearby catalytically important ionizing group. An objective of experiments now in progress is to delineate further the location of ionizing groups near or in the active site of papain in solution by means of the reaction of the enzyme with reagents related to I to produce covalently bound reporter groups in which phenol functions with different ionization constants are located at various distances from the sulfur atom of Cys-25. These experiments will aid in establishing the nature of the catalytically important groups whose ionizations are reflected in the pH–rate profiles which have been measured for papain.

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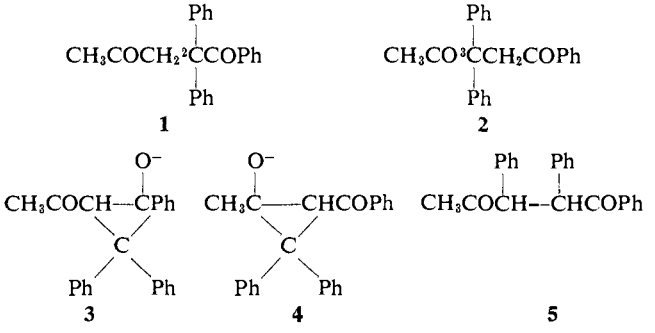
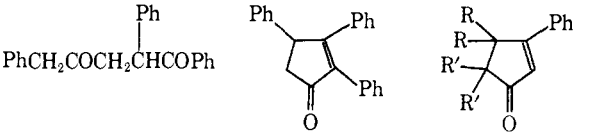
Mechanism of the Base-Catalyzed Interconversion of γ-Diketones

Sir:

The rearrangement of an enolate anion of the γ-diketone **1** to that of the γ-diketone **2**, recently reported from these laboratories, was considered to proceed *via* the two homoenolate ions **3** and **4**, rather than *via* two 1,2-phenyl migrations involving enolate anions of **5**.[1] We now present two pieces of evidence that confirm this view.

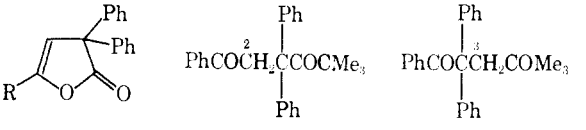


Treatment of deoxybenzoin in tetrahydrofuran with sodium hydride followed by 1-bromo-1-phenyl-2-propanone gave a mixture of the diastereoisomers of **5**, which were separated by fractional crystallization [**5a**: mp 153.5–154°; $\lambda_{max}^{CCl_4}$ 5.85, 5.96, 7.41 (m), 7.79 (w), 8.02 (w), 14.32 μ; λ_{max}^{EtOH} 247 mμ (ε 14,800); δ^{CDCl_3} 2.15 (s, 3 H), 4.60 (d, J = 11 Hz, 1 H), 5.20 (d, J = 11 Hz, 1 H), 7.3 (m, 13 H), 8.05 (m, 2H); **5b**: mp 184–185°; $\lambda_{max}^{CCl_4}$ 5.85, 5.96, 7.41 (m), 7.83, 14.35 μ; λ_{max}^{EtOH} 248 mμ (ε 13,200); δ^{CDCl_3} 1.92 (s, 3 H), 4.84 (d, J = 11 Hz, 1 H), 5.60 (d, J = 11 Hz, 1 H), 7.4 (m, 13 H), 7.9 (m, 2 H)].[2] Formed together with **5a** and **5b** were the diketone **6**,[3] mp 111.5–112° [$\lambda_{max}^{CCl_4}$ 5.86, 5.97 μ; λ_{max}^{EtOH} 246 mμ (ε 13,600); δ^{CDCl_3} 2.76 (dd, J = 4, 18 Hz, 1 H), 3.62 (dd, J = 10, 18 Hz, 1 H), 3.74 (s, 2 H), 5.13 (dd, J = 4, 10 Hz, 1 H), 7.3 (m, 13 H), 8.0 (m, 2 H)] and its dehydration product **7**, mp and mmp 139.5–140.5° (lit. mp 142–143°).[4] When **5a** was treated in ether with sodium methoxide[5] for 3 days, neither **1** nor **2** nor the corresponding cyclopentenones **8a** and **8b** could be detected in the reaction mixture,[6] demonstrating that enolate anions of **5** are not intermediates in the rearrangement of an enolate anion of **1** to that of **2**.



A critical difference between the two types of mechanism under consideration is that in the double homoenolate anion pathway the geminal phenyl groups remain attached to the same carbon atom (*i.e.*, C-2 of **1** becomes C-3 of **2**) while in the pathway involving two 1,2-phenyl migrations they do not and C-2 of **1** becomes C-2 of **2**. In order to establish by labeling experiments which of these relationships in fact holds, the analogous rearrangement of a different γ-diketone was examined in order to avoid both the complexity introduced by the occurrence of cyclopentenone formation and the inefficiency of the synthetic route to **1**.[1]

Reaction of the enol lactone **9**[7,8] with *tert*-butyllithium in ether at −15° for 2 min followed by work-up with aqueous acid gave the diketone **10** (80%), mp 130–130.5° [$\lambda_{max}^{CCl_4}$ 5.93 (br) μ; λ_{max}^{EtOH} 244.5 (ε 14,900), 280 mμ (sh, ε 1480), δ^{CDCl_3} 1.01 (s, 9 H), 4.18 (s, 2 H), 7.2–7.6 (m, 13 H), 7.8 (m, 2 H)]. Treatment of **10** in ether with sodium methoxide[5] for 7 days gave the rearranged diketone **11** (63%), mp 129.5–130° [$\lambda_{max}^{CCl_4}$ 5.85, 5.92 μ; λ_{max}^{EtOH} 235 (sh, ε 9500), 310 mμ (sh, ε 360); δ^{CDCl_3} 0.85 (s, 9 H), 3.81 (s, 2 H), 7.3 (m, 15 H)].[9] The structure of **11** was established by its independent synthesis in 85% yield by treatment of the enol lactone **12**[10] with phenyllithium in ether.



(1) P. Yates, G. D. Abrams, and S. Goldstein, *J. Amer. Chem. Soc.*, **91**, 6898 (1969).
(2) All melting points are uncorrected. Satisfactory elemental analyses have been obtained for all new compounds.
(3) This product could arise *via* isomerization of the bromo ketone, bromine transfer to the deoxybenzoin anion, or isomerization of the O-alkylation product; its origin is under investigation.
(4) C. P. Koelsch and T. A. Geissman, *J. Org. Chem.*, **3**, 480 (1938); P. Bladon, S. McVey, P. L. Pauson, G. H. Broadhead, and W. M. Horspool, *J. Chem. Soc. C*, 306 (1966). The cyclopentenone **7** was also formed when **6** was treated with methanolic sodium methoxide.
(5) The sodium methoxide was prepared *in situ* by the addition of sodium hydride followed by sufficient methanol to convert it to sodium methoxide.
(6) Much of **5a** was recovered unchanged together with several products formed in small amount; one of these was identified as **5b** on the basis of its R_f value. Under these conditions **1** gave a mixture of **8a** and **8b**.[1]
(7) P. Yates and T. J. Clark, *Tetrahedron Lett.*, 435 (1960); W. Reid and H. Mengler, *Justus Liebigs Ann. Chem.*, **651**, 54 (1962).
(8) Treatment of **9** with methyllithium gave **2**, thus confirming the earlier assignment[1] of the structure of the rearrangement product from **1**.
(9) The rearrangement of **10** to **11**, as of **1** to **2**, is essentially irreversible; this can be interpreted in terms of thermodynamic control resulting from steric effects.
(10) F. R. Japp and W. Maitland, *J. Chem. Soc.*, **85**, 1496 (1904).