Leaving Group Effects in Thiolester Hydrolysis. Part 2.† On the Possibility of an Elimination–Addition (Keten) Mediated Pathway in *S*-Acetylcoenzyme A Basic Hydrolysis and Acetyl Transfer

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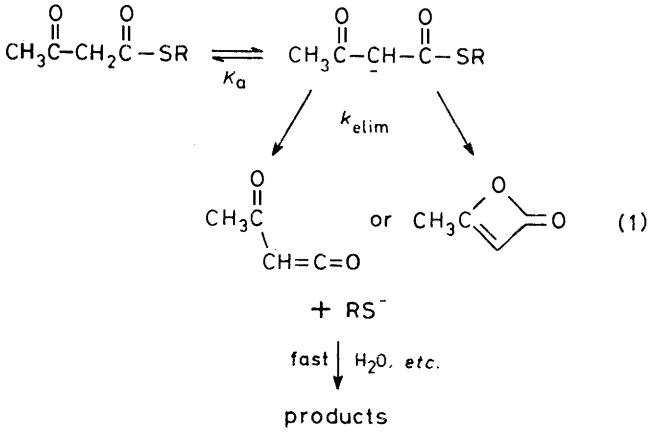
Alkaline hydrolysis rates (k_{HO^-}) at 25 °C in aqueous solution for a series of *S*-alkyl and *S*-aryl thiolacetates, including *S*-acetylcoenzyme A, were correlated (as their logarithms) with the pK_a of the conjugate acid of the thiolate leaving group to give a slope ($\beta_{l.g.}$) of −0.33. In comparison with the corresponding oxygen esters, thiolesters are, for the basicity of a given leaving species, one to two orders of magnitude less reactive towards hydroxide ion and show little dispersion into aryl and alkyl leaving groups, ascribed to the lower steric sensitivity of thiolacetate esters compared with the oxygen analogues. The small value of $\beta_{l.g.}$ and the lower reactivity of *S*- than *O*-esters are offered as evidence of a bimolecular associative ($B_{Ac}2$) mechanism for basic hydrolysis. The *E*2 route is excluded by the lack of deuterium incorporation into the (acetate) product of hydrolysis. In spite of the accepted acidity of thiolacetates, a kinetically insignificant amount of ester conjugate base is formed in aqueous solution even at high, non-physiological pH and thus *S*-acetylcoenzyme A does not hydrolyse by an *E*1cB pathway.

S-ACETOACETYLCOENZYME A hydrolyses [1] by means of an *E*1cB mechanism [equation (1)] in which the rapidly formed carbanion of this thiolester collapses in a unimolecular rate-determining step, a mechanism shared with other thiolacetoacetates [1] and aryl acetoacetates.[2]



(1)

Two of the necessary (but not sufficient) criteria for such a mechanistic possibility are a reasonably accessible conjugate base for a given substrate (a K_a effect) and an activated leaving group (a k_{elim} effect).

Whilst *E*1cB reactivity has been extensively studied [3-6] the amount of information available on leaving groups other than those based on oxygen is minimal. However, in spite of an inherently poorer leaving ability [1] for RS^- than RO^-, if judged on a p$K_{l.g.}$ basis,† of between 10^2- and 10^3-fold, *S*-acetoacetylcoenzyme A is still activated towards hydrolysis by an *E*1cB rather than a $B_{AC}2$ mechanism.[1] For this biologically important thiolester, the accessibility of the conjugate base is no limitation in basic or even neutral media as the pK_a values of acetoacetate esters [1,2] are in the range 7—10.

No only does the ester pK_a influence the mechanism by direct control of the amount of carbanion available in the reaction medium, but it is reflected mechanistically in an additional manner which is poorly understood currently. The rate of an *E*1cB process is a function both of the amount of anion present and of the intrinsic ability of that anion, once formed, to undergo elimination.[3-6] For a series of sulphonate esters, $HXSO_2OC_6H_4NO_2$-*p*, the rate of anion elimination (k_{elim}) depends markedly on the nature of X [see (A)].[5,7] The elimination order is X = PhCH ≫ MeN ≫ O and obeys an interesting linear free-

$$^-X\text{-}SO_2\text{-}OC_6H_4NO_2\text{-}p$$
(A)

energy relationship [equation (2)]. Thus, the more basic the 'internal nucleophile' (X^-), the more efficiently is

$$\log_{10}(k_{elim}/s^{-1}) = 0.9\ pk_{ester} - 10.5 \qquad (2)$$

the leaving group expelled.[7] This relationship is for a very wide range of structural types and may simply reflect the greater 'internal nucleophilicity' of a carbanion than of a nitrogen anion, *etc.* For a series of carbamate esters ($YNHCO{\cdot}OC_6H_5$) a similar trend has been reported.[8] Such observations may only be expected in so far as the rate of elimination (with kinetic formation of a π-bond) can parallel the equilibrium process for protonation.[4]

While the pK_a of acetoacetate esters is low [1,2] (*ca.* 7—10), acetate esters, without the activating β-acyl moiety, are considerably less acidic. However, the anion of a thiolacetate (*e.g. S*-acetylcoenzyme A) once formed would be expected to eliminate more rapidly than an acetoacetate anion.

Consequently, we have investigated the alkaline hydrolytic behaviour of thiolacetates, primarily to define mechanistic possibilities for and limitations on *S*-acetylcoenzyme A itself, which undergoes innumerable condensation reactions (at least formally involving the conjugate base) and acetyl transfer reactions *in vivo*.[9] Added impetus for such an investigation came from the common occurrence of thiolester intermediates in enzyme

† Where p$K_{l.g.}$ is the pK_a of the conjugate acid of the leaving group (*i.e.* RSH and ROH in this case).

mechanisms and the recent interest in the transition-states involved in thiolester transfer reactions.[10]

EXPERIMENTAL

Materials.—Deuterium oxide (99.8 atom % D) and [$^2H_3$]-acetonitrile (99 atom % D) were from Aldrich. *S*-Phenyl thiolacetate, obtained from Aldrich, was used without further purification as was *S*-acetylcoenzyme A, which was purchased as the sodium salt (90% assay) from Sigma. *S*-Acetylthiolcholine bromide was from Sigma. Other thiolacetate esters were synthesized from the appropriate thiol and acetic anhydride, under the catalytic influence of sulphuric acid, by the method of Böhme and Schran.[11] Esters were characterized by means of their physical properties, n.m.r. spectra and, for the new compounds, by elemental analysis. *S*-4-Methylphenyl thiolacetate showed b.p. 70° at 0.7 Torr (lit.,[12] 240—243° at 760 Torr). *S*-4-Methoxyphenyl thiolacetate showed b.p. 92—95° at 0.63 Torr (lit.,[13] 163—166° at 12 Torr. *S*-4-Chlorophenyl thiolacetate showed m.p. 37—38° (lit.,[14] 39—40°). *S*-4-Nitrophenyl thiolacetate showed m.p. 81.5—83.5° (lit.,[15] 81—82°). *S*-3-Nitrophenyl thiolacetate showed m.p. 61—63° (Found: C, 48.3; H, 3.5; N, 7.0. $C_8H_7NO_3S$ requires C, 58.7; H, 3.6; N, 7.1%).

Acetonitrile (Fisher Pesticide Grade) was further purified by molecular sieve (Linde 4A) treatment followed by distillation from a small quantity of phosphorus pentaoxide and finally by distillation from calcium hydride. All water used in kinetic studies was glass-distilled and degassed before stock solution preparations. Kinetics were studied in the presence of 10^{-5}M-disodium ethylenediaminetetra-acetate to minimize interference by metal ions. The ionic strength was held at 0.1 using sodium chloride as the support electrolyte unless otherwise stated.

Methods.—Rate measurements were performed either by spectrophotometric or pH-stat recording procedures. U.v.–visible spectrophotometry was carried out using a GCA-McPherson 707-K double-beam spectrophotometer, whose reaction chamber was thermostatted to 25.00 ± 0.02 °C by means of water circulating from a Haake E52 thermoregulator pump. Substrate (10–50 μl stock solution) was added on the flattened tip of a Teflon stirring rod to the appropriate medium (3.0 ml) equilibrated to 25 °C in a 10 mm path-length cuvette. The correct temperature equilibration was readily ascertained as the instrument was equipped with a thermocouple for immersion in the cuvette.

The Radiometer recording pH stat and titration system used consisted of a PHM 64 pH meter (±0.001 pH unit) with a TTT 60 titrator and REC 61 chart recorder. A calomel electrode was used in conjunction with a type G-2222B glass electrode in more basic media. The glass reaction vessel was kept at 25.00 ± 0.02 °C by means of a Thermomix model 1420 instrument. A type ABU 12 autoburette of 0.25 ml (for kinetic studies) or 2.50 ml (for measurement of dissociation constants) capacity was used to deliver titrant. All reactions were carried out under a stream of scrubbed, carbon dioxide-free, dry nitrogen. The pH meter was standardized against Fisher standard buffers: the titrant normality was checked against standard acid. Measured pH values were corrected, when necessary, for the sodium ion error.

Stock solutions of esters were prepared in acetonitrile except for *S*-acetylcoenzyme A, which was dissolved in water. Such solutions were prepared immediately before use.

Kinetics were studied by means of the pH-stat technique (under N_2) except for the 4-nitrothiophenyl (409 nm) ester and *S*-acetylcoenzyme A (232 nm) for which spectrophotometry was used.

N.m.r. exchange studies with 4-methoxyphenyl thiolacetate were carried out at 60 MHz in D_2O–CD_3CN (1 : 2) on an EM 360 spectrometer. Integrations of acetyl protons in substrate and base hydrolysis product were performed using the 4-methoxy-protons as internal reference standard.

M.p.s (uncorrected) were determined on an Electrothermal capillary melting point apparatus. Linear and exponential regression analyses were performed on a programmable Wang 720C calculator; we are grateful to the Department of Pharmacy at Duquesne University for use of the calculator and n.m.r. facilities.

RESULTS

Kinetics.—Alkaline hydrolysis of thiolacetate esters follows equation (3) with acyl–sulphur fission [16–18] although, in acidic media, alkyl–sulphur cleavage has been reported for trityl esters.[16,18] At high pH, therefore, the kinetics can be followed readily by titration of liberated protons or, in

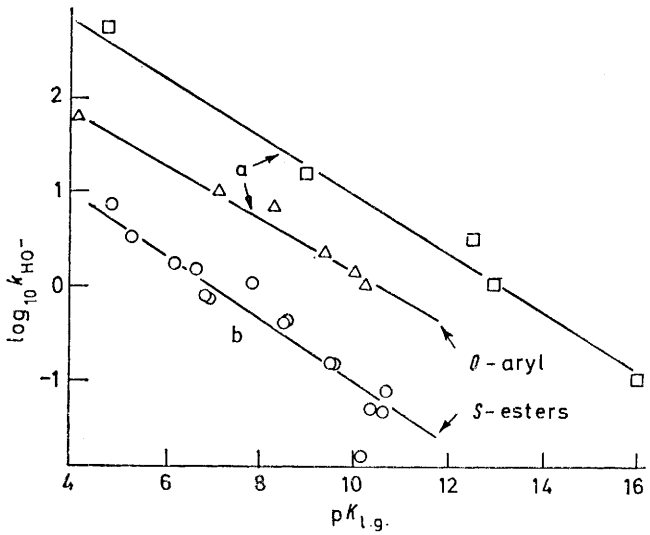
$$CH_3COSR + H_2O \xrightarrow{HO^-} CH_3CO_2H + RSH \quad (3)$$

suitable cases, by means of spectral changes associated with the reaction. Hydrolysis of the 4-nitrophenyl ester was followed by both methods ($\lambda_{kinetic}$ 409 nm) with consistent results. For this ester 98.6 ± 3.0% of the theoretical amount of 4-nitrothiophenolate ion was produced, based on an ε_M of 13 930 for this species.[19] Reactions were followed under pseudo-first-order conditions and plots of $\ln(A_\infty - A_t)$ *versus* time were linear to greater than 90% of reaction for the spectrophotometric studies; in pH-stat work analogous plots were linear to >85% of reaction.

The influence of small levels of acetonitrile co-solvent was small. At 0.01M hydroxide ion concentration the following rate constants were obtained for the 4-nitrothiophenyl ester (volume percentages of acetonitrile are in parentheses). 0.045 (6.23), 0.046 (3.54) and 0.048 s^{-1} (0.33). At 0.50% acetonitrile, the rate constant differs negligibly from the value (0.048 s^{-1}) obtained by computer-extrapolation to zero percent acetonitrile (r 0.999, linear regression analysis of k_{obs} *versus* percent acetonitrile). Rates were measured therefore in the presence of 0.5% (v/v) acetonitrile, except for *S*-acetylcoenzyme A for which no co-solvent was necessary.

The dependence of k_{obs} on hydroxide ion concentration was first-order for all esters up to the highest concentrations studied, which was 0.10M-HO^- for the 4-nitrophenyl ester using stopped-flow spectrophotometry. The linear dependence of k_{obs} on [HO^-] for 4-nitrothiophenyl acetate, which is most activated towards carbanion formation, indicates that no significant concentration of the ester anion is kinetically detectable even in 0.10M-NaOH; conjugate base formation would be expressed as downward curvature in such a plot.[3–6]

In the Table are collected the values of the second-order rate constants ($k_{HO^-} = k_{obs}/[HO^-]$) for hydroxide ion catalysed hydrolyses of the thiolacetates along with the pK_a (in water) of the conjugate acid of the corresponding thiolate leaving group. These data are compared in the Figure with analogous data from the literature [20] (at 25 °C and ionic strength 1.0) for the oxygen esters, CH_3COOR, in the form of a Brønsted plot for leaving group change *i.e.* $\log_{10} k_{HO^-}$ is plotted against pK_a (conjugate acid of leaving group). The



Brønsted-type leaving group plot for the dependence of the second-order rate constants (in l mol^{-1} s^{-1}) for hydroxide-ion attack ($\log_{10} k_{HO^-}$) on $pK_{l.g.}$ (the pK_a of the conjugate acid of the leaving group) for leaving-group substituted acetate esters, CH_3COXR: a, data for X = O from ref. 20; b, data for X = S from the Table. Lines are by least-squares linear regression analysis of the experimental data

thiolacetates, both aryl and alkyl, obey a linear free-energy relationship [equation (4)] wherein $pK_{l.g.}$ is the pK_a of the conjugate acid of the leaving group in water. The regression analysis was performed using all the points in the Table

Second-order rate constants (k_{HO^-}) for alkaline hydrolysis of thiolacetates, CH_3COSR in aqueous solution at 25 °C and ionic strength 0.1 in the presence of 0.5% v/v acetonitrile (except for *S*-acetylcoenzyme A which was studied in fully aqueous solution)

R	pK_{RSH} at 25 °C	k_{HO^-}/l mol^{-1} s^{-1}
$C_6H_4NO_2$-*p*	4.72 [a]	6.97
$C_6H_4NO_2$-*m*	5.24 [a]	2.95
C_6H_4Cl-*p*	6.14 [a]	1.63
C_6H_5	6.62 [a]	1.40 [b]
$C_6H_4OCH_3$-*p*	6.78 [a]	0.717
$C_6H_4CH_3$-*p*	6.82 [a]	0.677
$CH_2CH_2^+NMe_3$	7.80 [c]	1.10 [d]
CH_2CH_2NHAc	9.50 [c]	0.152 [e]
$CH(CO_2^-)NHAc$	9.52 [f]	0.123 [g]
coenzyme A	9.6 [h]	0.132
$CH(CO_2^-)CH_2CO_2$	10.09 [i]	0.0147 [g]
$CH_2CO_2^-$	10.25 [j]	0.0495 [g]
CH_2CH_3	10.50 [k]	0.0467 [e]
$CH_2CH_2CH_2CH_3$	10.66 [m]	0.0798 [g]

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save those for the thiocholine and thiomalate esters, which were twice as reactive and 6.5-fold less reactive than Brønsted prediction, respectively.

$$\log_{10} k_{HO^-} = 2.26\ (\pm 0.15) - 0.33\ (\pm 0.02)\ pK_{l.g.}\ (r\ 0.989) \quad (4)$$

A Hammett analysis of $\log_{10} k_{HO^-}$ *versus* σ^- constants yields equation (5).

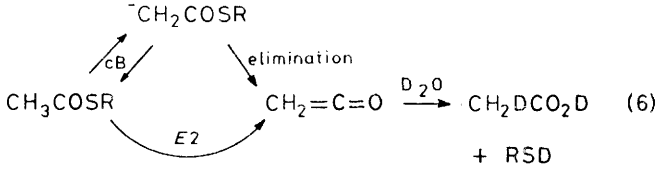
$$\log_{10} k_{HO^-} = 0.64\ (\pm 0.04)\ \sigma^- + 0.04\ (\pm 0.04) \quad (5)$$

N.m.r. Exchange Results.—Alkaline hydrolytic cleavage of 4-methoxythiophenyl acetate in 33% D_2O–67% CD_3CN medium at 0.125M-lyoxide ion concentration as studied by n.m.r. indicated that the acetate ion product had, within experimental error, incorporated no deuterium: the precision of the integration was such that incorporation of even a single deuterium atom per methyl group would readily have been detected.[21]

Dissociation Constant.—The pK_a of thiomalic acid was determined to be 10.09 ± 0.08 at 0.10M ionic strength in the presence of 10^{-5}M-EDTA at 25.0 °C (by Radiometer electrometric titration).

DISCUSSION

Possible elimination–addition (EA) mechanisms for the basic hydrolyses of thiolacetate esters analogous to *S*-acetylcoenzyme A, are given in equation (6).



(6)

The postulate of an EA mechanism for base-catalysed cleavage of thiolacetates has several pieces of evidence weighing against it. First, the *E*1cB mechanism involves a pre-equilibrium formation of carbanion and consequently when a protio-ester is solvolysed in D_2O one would observe rapid and essentially complete exchange of deuterium into the acetate product. Such rapid exchange for *bona fide* *E*1cB ester solvolyses has been reported.[3-6] However, we found no deuterium incorporation into the acetate product from the hydrolysis of 4-methoxythiophenyl acetate.[21] This observation also precludes the *E*2 mechanism, by means of which a single deuterium atom would be expected in the product. The value of $\beta_{l.g.}$ for the thiolacetates is low (−0.33) and close to that of the oxygen esters (Figure), a powerful counter-indication of an *E*1cB route for the base-catalysed hydrolysis of thiolacetates. *E*1cB Reactions of esters exhibit very high values of $\beta_{l.g.}$ (usually of the order of −1.2 to −2),[3-6] *e.g.* for aryl acetoacetates [2] $\beta_{l.g.}$ is −1.29, for *S*-aryl thiolacetoacetates [1] $\beta_{l.g.}$ is −1.13 and for the thiolcarbamate series PhNHCOSR we have found [22] $\beta_{l.g.}$ *ca.* −1.3.

The lower reactivity of *S*- than *O*-esters, on the basis of $pK_{l.g.}$ as the linear free energy parameter, further weighs against incorporation of a special ' keten route ' for the thiol esters. It is most likely that a bimolecular mechanism, analogous to that adopted by the oxygen esters is

followed by the thiolacetates, *i.e.* rate-determining attack of hydroxide ion on the ester carbonyl group. We must conclude that a kinetically significant amount of the enolate ion of the thiolacetate does not build up in aqueous solution. Even in 0.1M-NaOH there is no evidence of carbanion formation (Results) for the most activated of the thiolacetates. As elimination from ester carbanions has been shown to be considerably more rapid than from nitrogen-anionic conjugate bases of esters,[5,7] it is clear that the pK of the thiolester is considerably greater than that of the carbamate, under comparable aqueous conditions.

Although thiolesters are hydrolysed in basic solution with a considerably more negative free energy of hydrolysis than are oxygen esters,[23] it is believed generally that their rates of alkaline hydrolysis are comparable, *cf.* k_{HO^-} for ethyl acetate and ethyl thiolacetate which are 1.75 and 1.54 l mol^{-1} min^{-1} respectively at 20 °C in 62% acetone solution.[24] However, a significant difference exists between the members of this isostructural pair; mercaptans are more acidic than the structurally analogous oxygen counterparts. For any given basicity of leaving group, thiol esters are between one and two orders of magnitude less susceptible to hydroxide on attack than are oxygen esters (Figure). The source of this difference may lie in a real element effect or be an artefact in the sense that the p$K_{l.g.}$ may not be the most appropriate parameter to use for such a linear free-energy relationship (especially as the rate-determining step is undoubtedly attack of hydroxide ion on neutral ester). It is also notable that, although there is a major dispersion of oxygen esters into aryl and other leaving groups, no such marked dispersion is apparent for the thiolesters. The dispersion for *O*-alkyl and *O*-aryl esters has been ascribed to steric effects [25] and support for this hypothesis lies in our results[20] as *O*-esters are markedly more sensitive to steric effects in the leaving group than are *S*-esters,[24] *e.g.* for acetate esters, CH_3COXR, the ratio of k_{HO^-} for the methyl and t-butyl esters is 141 when X = O, but only 11 when X = S.[24]

The excellent fit of *S*-acetylcoenzyme A to the Brønsted plot indicates a bimolecular rather than a keten mechanism for its non-enzymic hydrolysis.

Lienhard and Jencks [25] have reported the rate of hydrolysis (5.9 l mol^{-1} min^{-1} at 25 °C) and the rate of deprotonation (k_1) of *S*-ethyl thiolacetate [equation (7)]. From the value of k_1, and the autoprotolysis constant or

$$CH_3COSEt + HO^- \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} {}^-CH_2COSEt + H_2O \quad (7)$$

water one can estimate the pK_a of this thiolester if the k_{-1} step is assumed [2,26] to be diffusion-controlled ($K_a = k_1K_w/k_{-1}$). This leads to a pK_a of *ca.* 26.4 for *S*-ethyl thiolacetate. From this the pK_a of *S*-acetylcoenzyme A can be estimated as *ca.* 26 if the dependence of ester pK_a shows a dependence on p$K_{l.g.}$ similar to that for the thiolacetoacetates [1] (β_{K_a} *ca.* 0.25—0.30). The work of Lienhard and Jencks also provides evidence against the operation of an EA route for *S*-ethyl thiolacetate as the observed rate of alkaline cleavage of the CO–S bond (k_{HO^-} 5.9 l mol^{-1} min^{-1}) is considerably faster than the rate of cleavage by hydroxide ion of the C–H bond (0.4 l mol^{-1} min^{-1}).

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