Mono[6-(2-mercaptoethylamino)-6-deoxy]-β-cyclodextrin as an Efficient Glyoxalase Mimic

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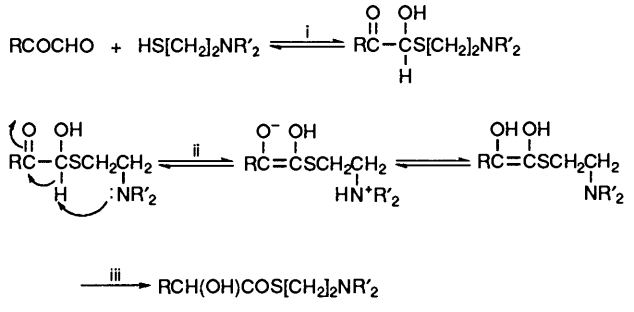
Trifunctional mono[6-(2-mercaptoethylamino)-6-deoxy]-β-cyclodextrin (MACD) has been prepared and used as a glyoxalase model. When 2-naphthylglyoxal (NAGO) is employed as a diagnostic substrate, MACD shows an overall activity greater than the reference compound 2-(dimethylamino)ethanethiol (DAET) at around pH 9; MACD favours remarkably the preequilibrium α-keto hemithioacetal formation, but slightly decelerates the follow-up rearrangement. The pH and kinetic isotope effects on the rate have revealed the mechanistic difference between the MACD- and DAET-promoted reactions.

Glyoxalases (I and II) and glutathione constitute a set of glyoxalase enzymes, which are of interest because of their importance in catalysing the transformation of α-keto aldehydes into the corresponding α-hydroxycarboxylic acids.[1] Since Franzen's discovery of an enzyme-model containing both amino and thiolate functionalities,[2,3] these enzymes have received considerable attention with regard to their mechanistic chemistry.



Scheme 1

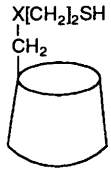
The transformation involves sequential formation of an α-keto semithioacetal, deprotonation to form the enediol, and reprotonation leading to the α-hydroxythiocarboxylate (Scheme 1). Okuyama and coworkers have undertaken a detailed kinetic investigation and have proposed a sophisticated mechanism in which the reaction includes a change of the rate-determining step depending on the reaction pH.[4] To our knowledge, however, no experiments have been reported aimed at designing an elaborate model system possessing both a selective binding site and a catalytic site. In this work, therefore, we have synthesised a cyclodextrin-based glyoxalase model and kinetically investigated the naphthylglyoxal to 1-naphthyl-1-hydroxyacetate transformation and have found that the model exhibits a mechanistically unique feature distinct from a simple model 2-aminoethanethiol.

Experimental

Materials.—Toluene-*p*-sulfonyl chloride was recrystallised twice from hexane prior to use. Pyridine was distilled from CaH immediately prior to use.

Mono[6-(*p*-tolylsulfonyl)-6-deoxy]-β-cyclodextrin (β-CD-OTs) and the corresponding iodide (β-CD-I) were synthesised according to the literature procedure with a minor modification, as follows.[5] β-CD vacuum-dried at 110 °C (10.0 g, 8.8 mmol) was allowed to react with toluene-*p*-sulfonyl chloride (2.0 g, 10.5 mmol) in 300 cm^3 pyridine for 24 h at room temperature. Evaporation of the pyridine under reduced pressure at 30 °C gave a white solid, which was washed with a copious amount of ether and then recrystallised twice from 300 cm^3 of water (80 °C). Filtration and vacuum-drying at 50 °C of the resultant white solid afforded 2.19 g (19.3%) of β-CD-OT: m.p. 170–180 °C (decomp.); δ(400 MHz; [$^2H_6$]DMSO) 2.48 (s, 3 H, Me), 3.2–4.5 (m, 63 H), 4.85 (s, 7 H, CH), 5.71 (m, 14 H, sec-OH), 7.45, 7.78 (all m, 2 H each, ArH) and 7.78 (m, 2 H, ArH). TLC on silica gel (PrOH–AcOEt–H_2O–NH_3 = 5:3:3:1) gave a single spot at R_f = 0.5, anisaldehyde positive. However, as HPLC on an ODS column (DMF–H_2O = 1:1) indicated that the product was contaminated with β-CD and pyridine, further purification was performed by HPLC (LC-908 Recycling Preparative HPLC, Japan Analytical Inc.) with the same developing solvent as described above. To HPLC-purified β-CD-OTs (2.0 g, 1.5 mmol) dissolved in 20 cm^3 of DMF was added powdered KI (2.57 g, 15.5 mmol). The mixture was kept at 80 °C for 24 h. After evaporation of the solvent, 40 cm^3 of water and 2 cm^3 of tetrachloroethylene were added with vigorous stirring. The resulting solid was filtered off, and dried at 50 °C for 24 h under reduced pressure, to give 1.60 g of β-CD-I; TLC on silica gel (PrOH–AcOEt–H_2O–NH_3 = 5:3:3:1) gave one spot at R_f = 0.5, anisaldehyde positive. Removal of the tetrachloroethylene was effected by dissolving the iodide in DMF and evaporating off the DMF under reduced pressure, to give 1.2 g of the iodide [Found: M, 1243.0 (FAB neg ion). Calc. for $C_{42}H_{69}O_{34}$: 1244]. The product was judged pure enough to proceed to the next step. Mono[6-(1,8-diaza-4,5-dithiaoctyl)-6-deoxy]-β-cyclodextrin was prepared by treating 1.2 g of β-CD-I with a 20-fold excess of water-free cystamine (2,2′-dithiobis-(ethylamine)) in 20 cm^3 of DMF at 40 °C for 2 days. Solvent removal from the mixture *in vacuo*, neutralisation with aq. Na_2CO_3, and then precipitating with tetrachloroethylene gave the desired disulfide: δ(400 MHz; [$^2H_6$]DMSO) 2.7–2.9 (m, 10 H, NCH_2 and 2 × NCH_2CH_2S), 3.1–3.8 (m, *ca.* 53 H), 4.80 (s, 7 H, CH). In order to remove the included tetrachloroethylene, the disulfide was dissolved in DMF and the DMF was distilled off. The tetrachloroethylene-free disulfide (0.50 g) thus obtained was reduced with a 10-fold excess of mercaptoethanol acting as both a solvent and reducing agent. The mixture was weakly acidified with a minimum quantity of dilute hydrochloric acid and then poured into 100 cm^3 of 20% H_2O–acetone under nitrogen to give a white precipitate. To avoid contamin-

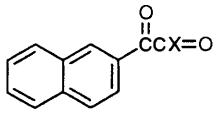
ation of the mercaptoethanol and 2-aminoethanethiol, the precipitate was redissolved in the minimum quantity of water and reprecipitated out from 5% H_2O–acetone, which provided the white powder mono[6-deoxy-6-(2-mercaptoethylamino)]-β-cyclodextrin (MACD) as the HCl salt in 85% yield: δ(400



X = NH_2^+ : MACD
S : MSCD

MHz; [$^2H_6$]DMSO) 3.1–3.8 (m, *ca.* 59 H), 4.80 (s, 7 H, CH). The SH content was assayed to be 0.98 ± 0.02 per molecule according to the Ellman method.[6]

2-Naphthylglyoxal (NAGO) was prepared by a literature method.[7] The crude oily light yellow product obtained by SeO_2-oxidation of 2-acetoxynaphthalene was purified by column chromatography on silica gel with $CHCl_3$ and then by recrystallisation from water containing a small amount of EtOH, to afford a total yield of white powders of 32%: m.p. 106–108 °C; δ([$^2H_6$]DMSO) 3.33 (s, 1 H, H_2O), 5.80 (t, 1 H, CH), 6.70 (d, 2 H, OH), 7.4–8.1 (m, 6 H, ArH) and 8.65 (br s, 1 H, ArH). The NMR spectrum in [$^2H_6$]DMSO showed that NAGO exists in the hydrated form together with a trace of H_2O-free NAGO, the methine peak of which appeared at 8.5 ppm. [^{2}H]NAGO (95% D) was synthesized by SeO_2-oxidation of 2-([$^2H_3$]acetoxy)naphthalene, which had been prepared by H/D exchange of 2-acetoxynaphthalene (5.4 g) in 18 cm^3 D_2O (99% D) and 20 cm^3 THF containing 0.2 equiv. NaOD as a catalyst at 80 °C for 24 h: δ(400 MHz; [$^2H_6$]DMSO) 3.33 (s, 1 H, H_2O), 6.70 (s, 2 H, OH), 7.4–8.1 (m, 6 H, ArH), 8.65 (br s, 1 H, ArH). 2-(Dimethylamino)ethanethiol (DAET) was purchased from Tokyo Kasei Co. (Japan) and used as received.



NAGO; X = H, D

Product Analysis.—To 10 cm^3 of an aqueous, pH 9 solution of DAET (5.6 g, 39.6 mmol) was added NAGO (800 mg, 3.96 mmol) in 2 cm^3 MeOH with stirring under nitrogen. After 24 h, UV monitoring showed complete reaction. The solution was then made alkaline with NaOH to pH 11 to hydrolyse the thioester product. After 2 days, acidification of the solution and then filtration of the precipitates afforded 720 mg of α-(2-naphthyl)-α-hydroxyacetic acid in 90% isolated yield: m.p. 160–162 °C; δ(60 MHz; [$^2H_6$]DMSO) 5.05 (s, 1 H), 7.3 (m, 3 H) and 7.7 (m, 4 H).[8] Meanwhile, a mixture of 10 mg of NAGO and a twofold excess of MACD in aqueous solution (pH 10.0) was allowed to react at ambient temperature for 2 days under nitrogen, and then, after alkaline hydrolysis of the thioester, the solution was weakly acidified with dilute hydrochloric acid. The yield of α-(2-naphthyl)-α-hydroxyacetic acid determined by HPLC (ODS column; MeCN–H_2O = 1 : 1 v/v) was 61%.

Kinetics.—The kinetics were measured at 25 °C and constant ionic strength (μ = 0.1 mol dm^{-3}; NaCl) under a nitrogen atmosphere. We felt it necessary to examine the reaction under simplified conditions without using buffered solutions, since the reaction is known to be subject to substantial general-base catalysis, which would complicate the kinetics. HCl or NaOH was added to obtain a desired pH value; we checked that the reaction pH value did not change significantly during the kinetic experiments. NAGO and DAET or MACD concentrations were 0.7 × 10^{-4} and 2.0–20 × 10^{-4} mol dm^{-3}, respectively. The reaction solutions were placed in a 1 cm cuvette under a nitrogen atmosphere. The capped cuvette was placed in a thermally regulated cell holder, and the run was monitored at appropriate pH values by following the decrease in absorption at 300 nm due to NAGO alone. Typical UV traces with time are displayed in Fig. 1. UV spectroscopic measurements were obtained on a Shimadzu UV-160A recording spectrophotometer. The ^{1}H NMR spectra were recorded using Nihon Densi spectrometer. Pseudo-first-order rate constants were calculated by means of the integrated first-order rate equation.

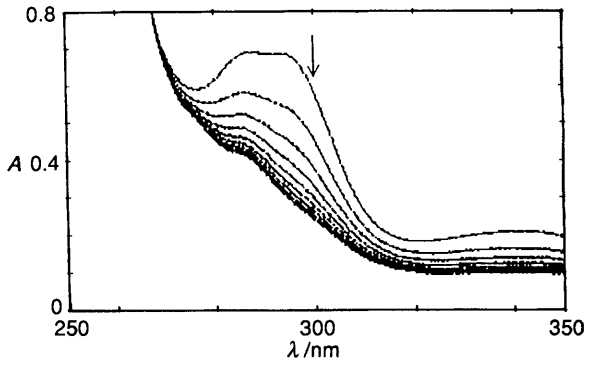


Fig. 1 Spectra relevant to the reaction of NAGO (0.7 × 10^{-4} mol dm^{-3}) with DAET (14 × 10^{-4} mol dm^{-3}) at 25 °C under N_2

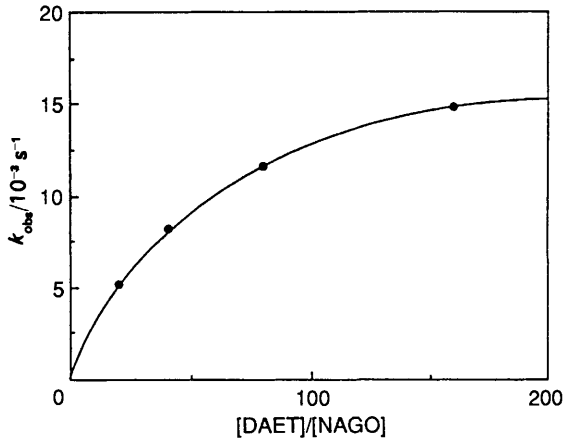


Fig. 2 Dependence of k_{obs} on [DAET] at pH = 9.75, 25 °C under N_2; [NAGO] = 0.7 × 10^{-4} mol dm^{-3}

Results and Discussion

Effect of Varying Thiol Concentrations.—Treatment of NAGO with an excess of aminoethanethiols (AET) resulted in decreased absorbance at 300 nm due to NAGO, and the NMR spectra in [$^2H_6$]DMSO of the reaction mixtures exhibited a peak at 5.2 ppm which was attributable to the production of the rearranged α-hydroxythiocarboxylate in excellent yields.[4] Throughout this work the rates were measured without the use of buffer in order to avoid complications caused by possible general acid–base catalysis by a buffer system. General acid–base catalysis by AET itself was negligible since its concentration was too low. The effects of changing amino thiol concentrations in aqueous solutions at constant pH 9.75 were examined and the results for DAET and MACD are displayed in Figs. 2 and 3, respectively. The results indicate that MACD is more effective in promoting the reaction rate than is DAET at the low concentrations in which they were present. Each

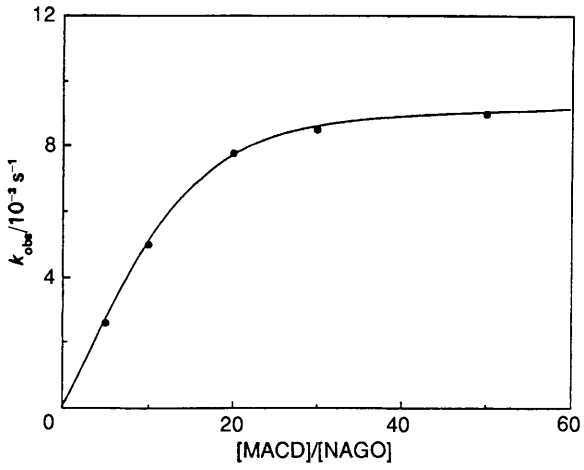


Fig. 3 Dependence of k_{obs} on MACD concentration at pH = 9.75, 25 °C under N_2; [NAGO] = 0.7 × 10^{-4} mol dm^{-3}

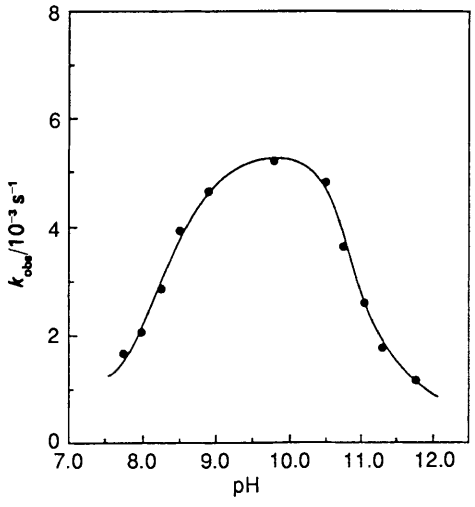


Fig. 4 pH–rate profile for the DAET-catalysed NAGO rearrangement at 25 °C under N_2; [NAGO] = 0.7 × 10^{-4} mol dm^{-3}, [DAET] = 14 × 10^{-4} mol dm^{-3}

observed rate constant, k_{obs}, reached a maximum with increasing aminoethanethiol concentrations, implying that the reaction involves a fast bimolecular process followed by a rate-limiting process. Thus, the glyoxal transformation may be described by eqns. (1) and (2) which provides the kinetic

$$\underset{\text{(NAGO)}}{\text{NpCOCHO}} + \underset{\text{(AET)}}{\text{R}_2\text{N(CH}_2)_2\text{SH}} \overset{K_{hemi}}{\rightleftharpoons} \underset{\text{(HEMI)}}{\text{NpCOCH(OH)SCH}_2\text{CH}_2\text{NR}_2} \quad (1)$$

Np = naphthyl

$$\text{HEMI} \xrightarrow{k_{rear}} \text{NpCH(OH)CO(SCH}_2\text{CH}_2\text{NR}_2) \quad (2)$$

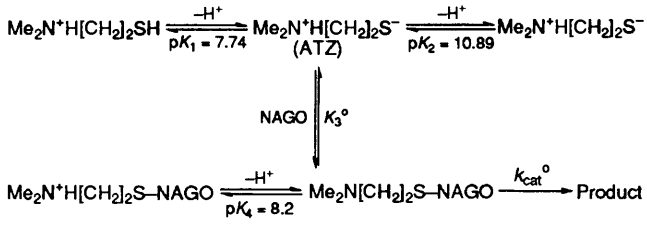
expression in eqn. (3) when the kinetics were measured under

$$v = -\text{d[NAGO]}/\text{d}t = k_{obs}[\text{NAGO}] \quad (3)$$

$$k_{obs} = k_{rear}[\text{AET}]_0/(K_{hemi} + [\text{AET}]_0)$$

conditions of $[\text{AET}]_0 \gg [\text{NAGO}]$. The validity of the above treatment was confirmed by the fact that the reaction is strictly first order in the NAGO concentration for more than three half-lives throughout. The Lineweaver–Burke plots of these data points gave k_{rear} = 20 × 10^{-3} s^{-1}, $1/K_{hemi}$ = 250 dm^3 mol^{-1} for DAET and k_{rear} = 12 × 10^{-3} s^{-1}, $1/K_{hemi}$ = 1100 dm^3 mol^{-1} for MACD at pH 9.75. Thus, there is significant difference in binding constant, $1/K_{hemi}$, between MACD and DAET, the value for the former being more than six times greater than that for the latter. This implies that the binding of the naphthyl moiety in the hydrophobic β-CD cavity facilitates α-keto semithioacetal formation.

pH Effect.—The pH effect on rate reflects important information concerning a reaction mechanism. In order to analyse the pH profile, the kinetics of the DAET-catalysed rearrangement of NAGO were measured as a function of the DAET concentration at several pH values. The results for DAET are summarised in Fig. 4, which shows a bell-shaped pH–rate profile with the optimum pH of 9.8. Generally, such a bell-shaped profile can be accounted for by participation of two or more protons as shown in Scheme 2,[9] where, of three kinetically important forms of an amino thiol, only the electrically neutral species such as ammonium thiolate zwitterion (ATZ) is considered to be responsible for the α-keto semithioacetal formation.[4,9]



Scheme 2 pK_1 and pK_2, ref. 9; pK_4, estimated in this work

The relevant equilibrium and kinetic constants were calculated based on the sequence in Scheme 2, which gives eqns. (4), (5) and (6).[10] Eqn. (4) could be reduced further to the simple forms (7a) and (7b) corresponding to relatively high and low

$$v = k_{cat}[\text{NAGO}][\text{DAET}]/(K_3 + [\text{DAET}]) \quad (4)$$

$$k_{cat} = k^{\circ}_{cat}/(1 + [\text{H}^+]/K_4) \quad (5)$$

$$k_{cat}/K_3 = (k_{cat}^{\circ}/K_3^{\circ})/\{1 + [\text{H}^+]/(K_1K_2/[\text{H}^+])\} \quad (6)$$

$$v = k_{cat}[\text{NAGO}] = k_{obs}[\text{NAGO}] \quad (7a)$$

$$v = k_{cat}[\text{NAGO}][\text{DAET}]/K_3 = k_{obs}[\text{NAGO}] \quad (7b)$$

DAET concentrations, respectively. The kinetic constants k_{cat}°, K_1, K_2, $k_{cat}^{\circ}/K_3^{\circ}$, K_4 were determined by varying their values until the fit to the experimental data is optimized: k_{cat}° = 2 × 10^{-2} s^{-1}, $k_{cat}^{\circ}/K_3^{\circ}$ = 3.9 dm^3 mol^{-1} s^{-1}, K_1 = $10^{-7.95}$ mol dm^{-3}, K_2 = $10^{-11.1}$ mol dm^{-3}, K_4 = $10^{-8.2}$ mol dm^{-3} for DAET. Thus, the observed kinetic basicities of the active sites (pK_1, pK_2 and pK_4) upon which the reaction rate depends are 8, 11 and 8.2, respectively; the K_1 and K_2 values thus obtained are consistent with the reported values.[9] However, the observed kinetic pK_4 of 8.2 seems rather low for the real pK_a value (*ca.* 10) expected for the DAET–NAGO semithioacetal complex; that is, the pK_a thus obtained does not represent a real ionization. Such a perturbed pK_a appears to be due to the reaction involving a change of the rate-determining step (Scheme 1);[11] that is, at high pH, the protonation [step (iii)] rather than deprotonation [step (ii)] becomes partially rate-determining. In fact, a smaller kinetic isotope effect was observed at pH > 10.5 (Table 1). The same type of perturbation of kinetic pK_a has been reported by Okuyama and his coworkers for *N*-(2-mercaptoethyl)piperidine-catalysed rearrangement of phenylglyoxal.[4] In conclusion, it is found that plots of k_{obs} values against pH at a constant

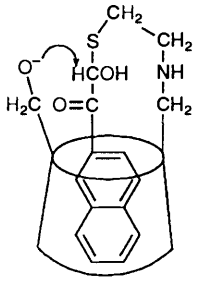
Table 1 Substrate deuterium kinetic isotope effect[a] at various pH

DAET		MACD	
pH	k_H/k_D[b]	pH	k_H/k_D[b]
8.50	5.6	8.10	2.5
9.78	5.3	10.00	2.4
10.75	4.2	10.92	2.3
—	—	11.49	1.8
—	—	11.91	1.0
—	—	12.25	1.0

[a] Conditions: 25 °C under N_2; [DAET] = [MACD] = 14 × 10^{-4} mol dm^{-3}; [NAGO] = 0.7 × 10^{-4} mol dm^{-3}. [b] Error estimated to be ± 5%.

[DAET] gives bell-shaped curves; the rate-depression in the pH region < 10 may be attributable to unproductive formations of the protonated covalent complex $Me_2N^+H(CH_2)_2S$–NAGO at high [DAET] and the protonated aminoethanethiol $Me_2N^+H(CH_2)_2SH$ at low [DAET] while a rate depression in the higher pH region is somewhat complex, but may be attributable to an accumulation of $Me_2N(CH_2)_2S^-$, which is virtually inactive for thioacetal formation, or to a change of the rate-determining step to enediol protonation.

The reaction of MACD in buffered solution shows progressive deviation with time from first-order kinetics. In particular, the extent of the deviation becomes remarkable in the very high pH region > 11.5. On the other hand, the reaction in the absence of buffer follows well-defined kinetics. Fig. 5 (○) shows that, as compared with the DAET case, the rate maximum is shifted greatly to pH 11.5, which prompts the suggestion that, with MACD, the hydroxyanion on the primary face of β-CD appears to be more favourable as the proton-abstracting base than the amino nitrogen in the pendant group. In Fig. 5 (●) is also included the kinetic results with mercaptoethanethio-substituted CD (MSCD) in which a divalent sulfur replaces the NH group in the pendant unit. Although divalent sulfur, unlike the basic amino nitrogen, will show virtually no affinity for protons, MSCD can catalyse the reaction. The pH–rate profile with the optimum pH of 11.5



Inclusion complex between MACD and NAGO

provides strong evidence to indicate that the thioacetal methine proton is fixed close to the ionized primary rim-hydroxy groups that take part exclusively in proton abstraction at high pH. In accordance with this interpretation, no comparable rate acceleration was observed with a simple sulfide such as mercaptoethanol the hydroxy group of which is not held fixed.

Kinetic Deuterium Isotope Effect (KIE).—In order to assess the kinetic importance of the C–H bond cleavage step [deprotonation step (ii) in Scheme 1], the substrate deuterium isotope effect k_H/k_D was determined as a function of pH in the presence of a large excess of aminoethanethiols. The results are summarised in Table 1; use of deuteriated NAGO as the substrate resulted in a more than five-fold decrease of the reaction rate for DAET except in the higher pH region where a

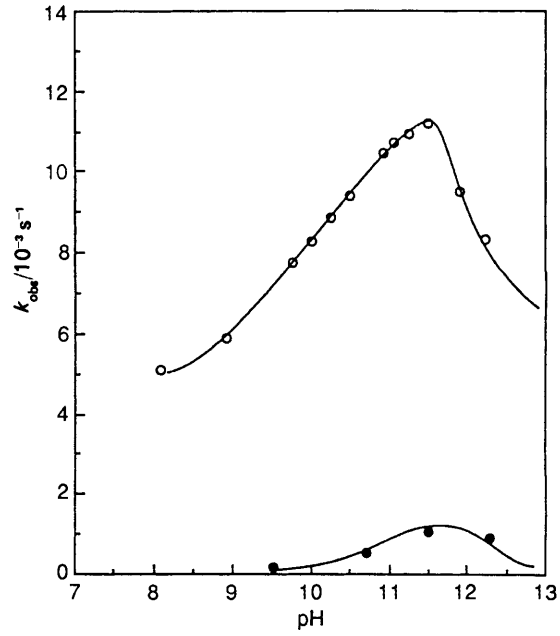
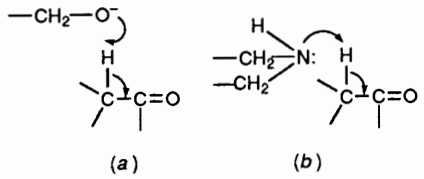


Fig. 5 pH-rate profile for the MACD (○) and MSCD (●)-catalysed NAGO rearrangement at 25 °C under N_2; [NAGO] = 0.7 × 10^4 mol dm^{-3}, [cat] = 14 × 10^{-4} mol dm^{-3}

change of rate-determining step might occur. This large rate-depression is indicative of C–H bond cleavage by a nitrogen-base being included in the rate-limiting step, and the large KIE value suggests a mid-point transition state for proton transfer to nitrogen.

Meanwhile, a KIE as small as 2 was observed in the MACD-reaction at pH < 11.5. This is a clear suggestion of mechanistic difference between the MACD- and DAET-reactions, namely that the small KIE might be due to a shift in the position of the transition state of the deprotonation along the reaction co-ordinate. According to the Hammond rule,[12] an O–H bond formation in the transition state will be less developed, *i.e.*, an early transition state, than the corresponding N–H bond formation, since O–H bond energy is far greater than N–H bond energy. Accordingly, a smaller KIE was observed in the MACD-reaction than in the DAET-reaction. Particularly interesting is the fact that no kinetic isotope effect was observed when pH > 11.5. This means that the rate-determining step changes from the deprotonation of the thioacetal [step (ii) in Scheme 1] to the subsequent reprotonation of the enediol [step (iii)].

Transition-state Structures for Deprotonation.—It becomes evident that the results of the pH effect on the MACD-reaction rate mentioned above is not compatible with the generally accepted mechanism in which proton abstraction by the intramolecular nitrogen base is included. Therefore, we propose the alternative possibility that the rim hydroxy anion located in the vicinity of the included naphthyl α-keto semithioacetal moiety is responsible for proton abstraction at a high pH; inspection of space-filling models reveals that the naphthyl group is held rigidly in the β-CD cavity, with the amino nitrogen and the hydroxy oxygen being forced into close proximity at an equal distance from the proton to be abstracted. However, a question arises as to why the C–H proton is attacked preferentially by the rim-hydroxy anion than by the pendant amino nitrogen. Quantum chemical theory predicts that a C–H bond undergoing cleavage must be parallel to the adjacent C–O π-orbital during deprotonation to maintain the π-conjugation between them. When this requirement is imposed on the transition-state structure in question, a careful inspection of



(*a*) (*b*)

Fig. 6 Transition states for proton-abstraction by oxygen (*a*) and nitrogen (*b*) bases in β-CD-hemithioacetal

space filling models leads us to conclude that deprotonation by the hydroxy anion is considered more likely, since the hydroxy oxygen atom can be in alignment with the C–H bond [Fig. 6(*a*)]. On the other hand, the amino nitrogen atom participates in non-linear N–H–C geometry with an internal angle as small as 90° which is an absolutely unfavourable geometry for proton transfer [Fig. 6(*b*)]. In other words, the included naphthyl unit greatly disturbs the interaction between the N and H atoms. However, we are unable to rule out the possibility that deprotonation by the nitrogen base contributes to the rate acceleration to an insignificant extent.

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