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ELUCIDATION OF THE MULTIPLE EQUILIBRIA OF MALVIN IN AQUEOUS SOLUTION BY ONE- AND TWO-DIMENSIONAL NMR

HELENA SANTOS,*† DAVID L. TURNER,‡ JOÃO C. LIMA,* PAULO FIGUEIREDO,† FERNANDO S. PINA† and ANTÓNIO L. MAÇANITA*§

*Centro de Tecnologia Química e Biológica, Apt 127, 2780 Oeiras, Portugal; †Departamento de Química, Faculdade de Ciências e Tecnologia, UNL, 2825 Monte de Caparica, Portugal; ‡Chemistry Department, University of Southampton, Southampton SO9 5NH, U.K.; §Departamento de Química, Instituto Superior Técnico, Lisboa, Portugal

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Abstract— One- and two-dimensional NMR were used to characterize the several forms of malvin present in aqueous solution in the pH range 0.3–4.5 and to determine their molar fractions as a function of pH. In addition to the flavylium cation, two hemiacetal forms and both the *cis* and *trans* forms of chalcone were firmly identified. The pathways for the interconversion of the different forms were derived and the molar absorbances of the species calculated by coupling NMR and UV/Vis data. Equilibrium constants were determined at different temperatures, and enthalpy and entropy changes were calculated for the interconversion processes. The conclusions are supported by molecular orbital calculations.

INTRODUCTION

Malvin (malvidin 3,5-diglucoside) is a water-soluble anthocyanin. Anthocyanins are largely responsible for the colours of flower petals and fruits and play an important role in the attraction of insects as pollination and seed dispersal agents; furthermore, they seem to be interesting as safe colour additives in the food industry [1] and this potential application led to considerable research efforts aiming to improve anthocyanin stability in aqueous solution.

The structural changes occurring in aqueous solutions of anthocyanins in the pH range 2–5 have been studied in detail by Brouillard *et al.* [2, 3] using spectrophotometric and fluorescence techniques. In the particular case of malvin, besides the flavylium cation (AH^+), the existence of two quinonoidal basic forms, which are tautomers derived from the cation by deprotonation, and hemiacetal and chalcone forms, which are related to the cation by the addition of hydroxide, have been proposed [3].

Nuclear magnetic resonance (NMR) is a powerful technique, not only for the identification of species in solution, but also for the characterization of exchange processes, provided that the exchange rates fall within the accessible range (*ca* $10^{-2}-10^{-4}$ sec^{-1}). Although NMR has been used extensively to determine the chemical structures of anthocyanins in solution (for comprehensive reviews see refs [4, 5]), these studies deal mostly with single species and the potential of NMR to study multiple equilibria of anthocyanins has not been fully exploited.

In the present study, one- and two-dimensional NMR were used to characterize the several forms of malvin which coexist in slightly acidic aqueous solutions; in particular, two-dimensional exchange correlation spectra provided crucial information about the structural transformations. The system was analysed over a range of pH and temperature values, and equilibrium constants and relevant thermodynamic parameters were determined for the interconversion processes. This information allowed the molar absorbances of the individual forms to be determined. The conclusions are further supported by visible spectroscopy and molecular orbital calculations.

RESULTS AND DISCUSSION

Assignment of species

The assignment of the resonances due to the flavylium form was made from the spectrum of a malvin sample at pH* (direct meter reading) approximately 0.87, where this is the only detectable form in solution (spectrum d in Fig. 1). Assignment of the resonance due to protons 2′ + 6′ follows immediately from the relative intensity of two protons expected for these equivalent nuclei. Proton 4 was assigned according to the literature [4] and proton 6 was assigned on the basis of an observed Nuclear Overhauser Effect (NOE) to the anomeric proton of one of the glucosidic moieties (Fig. 2). Proton 8 is assigned by the observation of a splitting of 2.5 Hz due to long-range scalar coupling to proton 6, and they both disappear from the spectrum in D_2O after a few days, a fact that indicates the more acidic character of these two protons.

The assignment of the several species interconverting in solution is then immediate from the two-dimensional exchange correlation spectrum (Fig. 2). Since we are

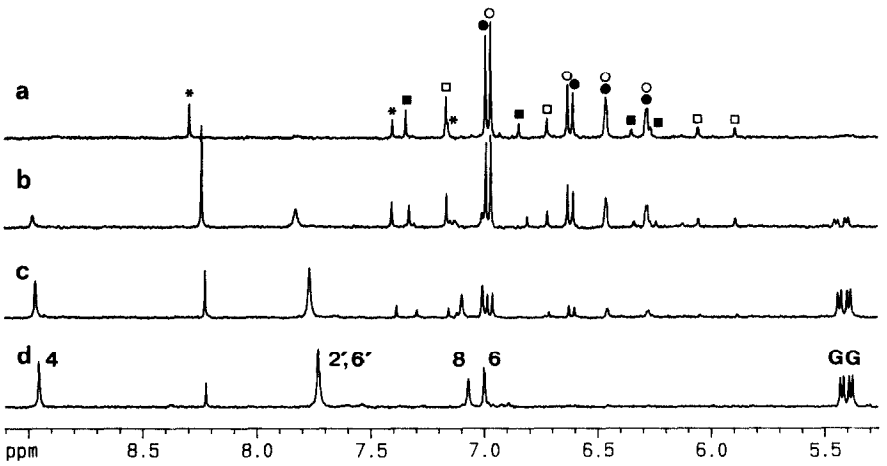

Fig. 1. ^{1}H NMR spectra (500 MHz) of malvin at a concentration of *ca* 1 mM in D_2O at different pHs* and $T = 298$ K; (a) pH* = 3.31, (b) pH* = 2.46, (c) pH* = 1.67, (d) pH* = 0.87. The flavylium peaks are labelled with their specific assignments in spectrum d; G indicates the glucose anomeric protons. The peaks of the remaining forms are indicated in spectrum a with the symbols: (●,○) *R/S* hemiacetal; (□) *cis*-chalcone; (■)*trans*-chalcone; (*) impurity.

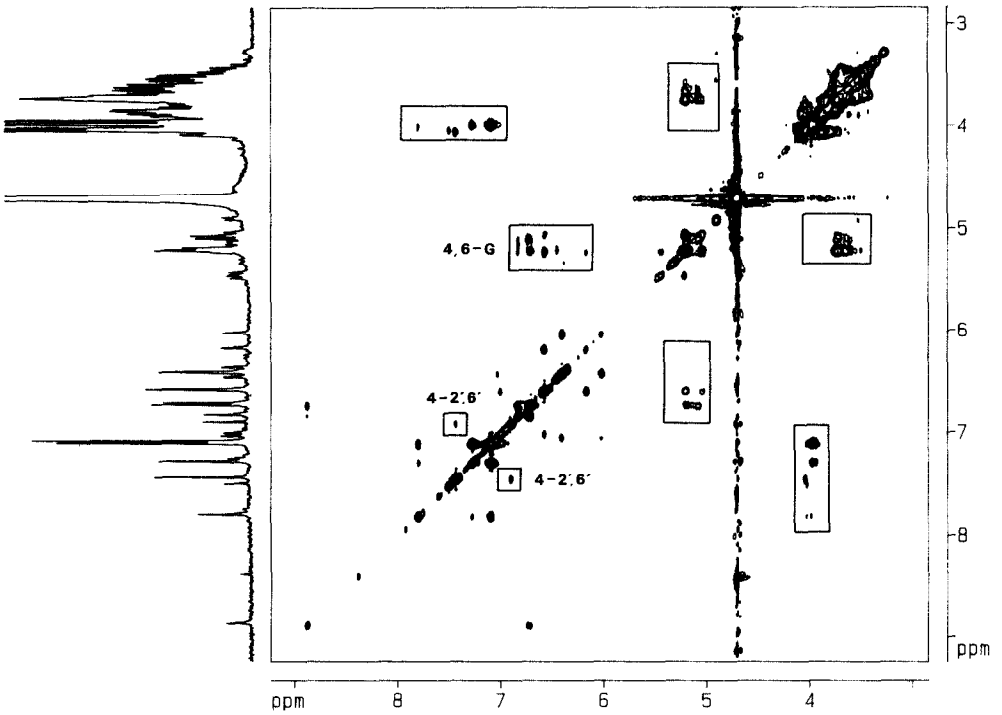

Fig. 2. NOESY spectrum (300 MHz) of a 0.8 mM malvin solution in D_2O at 313 K. The mixing time was 1.5 sec. Negative peaks are enclosed in boxes and are due to NOEs.

dealing with a small molecule and conditions of fast rotational diffusion hold, cross-peaks due to exchange are easily distinguished from those due to NOEs since they show intensities of opposite sign. Cross-peaks due to NOEs are negative and they are enclosed by boxes in Fig. 2. Negative NOEs (leading to positive NOESY cross-peaks) have been observed in one-dimensional spectra of concentrated solutions of several anthocyanidin 3,5-di-glucosides, where extensive aggregation was shown to occur [6]. The positive NOEs observed in our work are characteristic of fast molecular rotation and indicate that there is no aggregation.

Five chemical species are easily recognized in the one- and two-dimensional spectra in Fig. 2. Resonances due to the flavylium cation are already assigned and the remaining four forms have the same level of protonation, as evidenced by the pH dependence of the respective concentrations (Fig. 4 and discussion below). Furthermore, the

fact that long-range couplings between protons 6 and 8 are detected in these four forms immediately suggests that their structures are not widely different. The flavylium cation peaks show strong exchange connectivities to two other species, e.g. H_4 at 8.97 ppm has cross-peaks with resonances at 6.60 and 6.62 ppm (Fig. 2), and the proton assignments of several resonances follow directly from these connections. Both of these forms are connected by exchange to a further form, which also has much weaker cross-peaks to the flavylium cation. This suggests that the first two are intermediates in the interconversion of flavylium and the third form. Resonances due to the fourth neutral species are sharper and do not show any connectivities via exchange; therefore, a much slower interconversion rate must hold in this case. However, there is a clear NOE connection between the resonance due to H_4 and the resonance due to $H_2' + H_6'$ which is specific to this form which, together with the absence of NOEs between H_4 and the anomeric glucose protons allows its assignment to the *trans*-chalcone. Thus, it is concluded that the rate of interconversion of *trans*-chalcone with any of the other species is very slow (less than 0.01 sec^{-1}).

The sets of resonances of the two predominant forms have very similar chemical shifts, intensities and NOEs to glucose moieties, and the ratio of intensities does not change with pH. Also, the associated UV/Vis absorption spectra ($\lambda_{max} = 280$ nm) show that the rings are not conjugated and thereby they are assigned to the *R* and *S* isomers of the hemiacetal. There is insufficient information to determine which resonances arise from which isomer and they are simply labelled hemiacetal 1 and hemiacetal 2. The isomerization rate is too slow to detect (less than 0.01 sec^{-1}) since no cross-peaks connecting these two forms were observed and probably involves either the flavylium ion or a chalcone form as an intermediate.

The remaining form exhibits a set of resonances that parallel those of the *trans*-chalcone and is assigned to the *cis*-chalcone. The chemical shifts of the aromatic protons of the five forms of malvin are listed in Table 1. The data show no evidence for the presence of a basic form, which would result from simple deprotonation of the flavylium cation, in contrast with conclusions reached by other authors concerning malvidin 3-glucoside [7]. In fact, the pH dependence of the chemical shifts of the resonances due to the flavylium form is at most 0.1 ppm and a significantly larger change would be expected if a fast exchange between the acidic and basic forms was occurring. Furthermore, no peaks assignable to other species are observed in the spectra for the investigated pH* range (< 4.5), other than impurities which are not involved in the equilibria and which appear to result from irreversible degradation of malvin.

A scheme representing the reaction pathway in accordance with the present NMR results is presented in Fig. 3.

pH dependence of the concentrations of the several forms

The pH dependence of the concentrations of the five identified species was monitored by proton NMR at two temperatures: 298 K and 313 K. A selection of spectra at different pH* values and T = 298 K is shown in Fig. 1 and the corresponding values measured for the molar fractions are presented in Fig. 4. The curves are best fits to the experimental points and the fitted equilibrium constants for relevant equilibria are as follows: for the flavylium cation form and the hemiacetal forms, $K = 0.88$; for the *R* and *S* hemiacetal forms $K = 0.88$; for the *R/S* hemiacetal forms and the *cis*-chalcone, $K = 0.21$; and for the equilibrium between *cis*- and *trans*-chalcone, $K = 0.56$. The effect of temperature on the equilibrium constants was obtained from measurements of the temperature dependence of the resonance intensities in NMR spectra of a malvin solution at pH* 2.6. The enthalpy and entropy changes for the various isomerization reactions were calculated from appropriate Van't Hoff plots (Fig. 5) and are shown in Table 2.

Calculation of UV/Vis molar absorbances for the malvin forms

Absorption spectra of malvin in H_2O, at several pH values are shown in Fig. 6. The molar absorbances of two of the species are obtained directly by using the values of the respective concentrations at equilibrium as determined by NMR: flavylium cation, $\varepsilon_{AH^+}(520\text{ nm}) = 25\,600$ $l\,mol^{-1}\,cm^{-1}$ [8]; hemiacetals, $\varepsilon(275\text{ nm}) = 15\,400\,l\,mol^{-1}\,cm^{-1}$. The first value is in reasonable agreement with that previously reported [1]. In the case of the chalcone isomers the determination is not as straightforward because it is not valid to assume identical ε values for both forms. Furthermore, the absorption spectrum of the HPLC isolated *trans*-chalcone isomer is practically identical to that of the *cis*-chalcone isomer due to the band overlap of this latter isomer with the hemiacetal form (Lima, J. *et al.*, unpublished results). However, it was possible to obtain the separated absorption spectra of the two isomers from the fluorescence excitation spectra

Table 1. *Chemical shifts of the aromatic resonances of the five forms of malvin detected in* D_2O *at* 25° and pH* = 2.6

	Flavylium	Hemiacetal 1	Hemiacetal 2	*cis*-Chalcone	*trans*-Chalcone
H_4	8.97	6.62	6.60	6.71	6.81
$H_2' + H_6'$	7.82	6.99	6.96	7.16	7.33
H_6	7.00	6.46	6.46	6.05	6.33
H_8	7.10	6.28	6.28	5.89	6.24

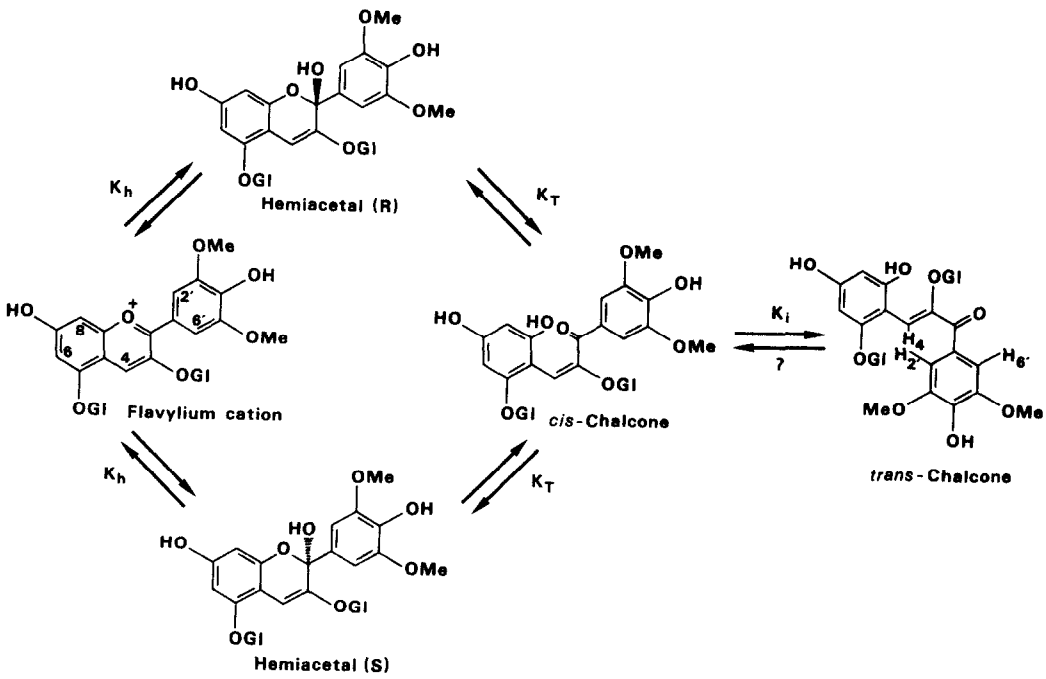

Fig. 3. Structure of the various forms of malvin. The reversible reactions detected by NMR are shown. The question mark indicates that the pathway to *trans*-chalcone was not determined.

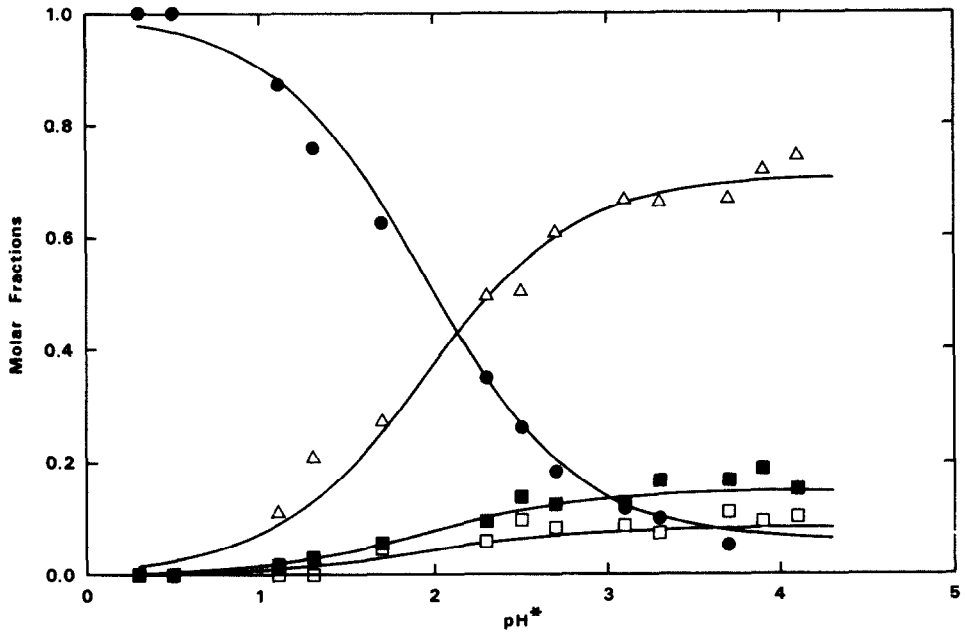

Fig. 4. Plot of the molar fractions of the various forms of malvin detected by NMR as a function of pH* and at $T = 298$ K. The data refer to the experiment illustrated in Fig. 1. (●) Flavylium cation; (△) hemiacetal forms (sum); (■) *cis*-chalcone; (□) *trans*-chalcone.

collected at emission wavelengths where only one of the isomers emit. The fractional absorbance of each isomer can be evaluated by fitting the weighted sum of these distributions to the total chalcone absorption band. Using the concentrations determined by NMR one obtains: ε_{cis} (328 nm = 10200 l mol^{-1} cm^{-1} and ε_{trans} (338 nm) = 18900 l mol^{-1} cm^{-1}. These results were confirmed by using the fact that ε is nearly temperature independent when the appropriate density corrections are made and repeating the fitting at two temperatures.

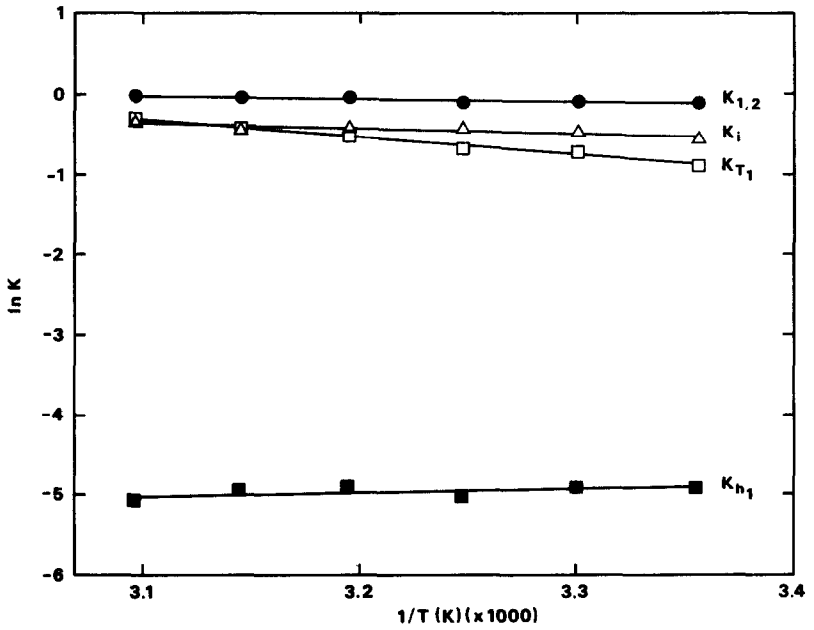

Fig. 5. Logarithm of the equilibrium constants as a function of the reciprocal temperature. The indexes 1 and 2 refer to the two hemiacetal isomers. Other indexes as in Fig. 3.

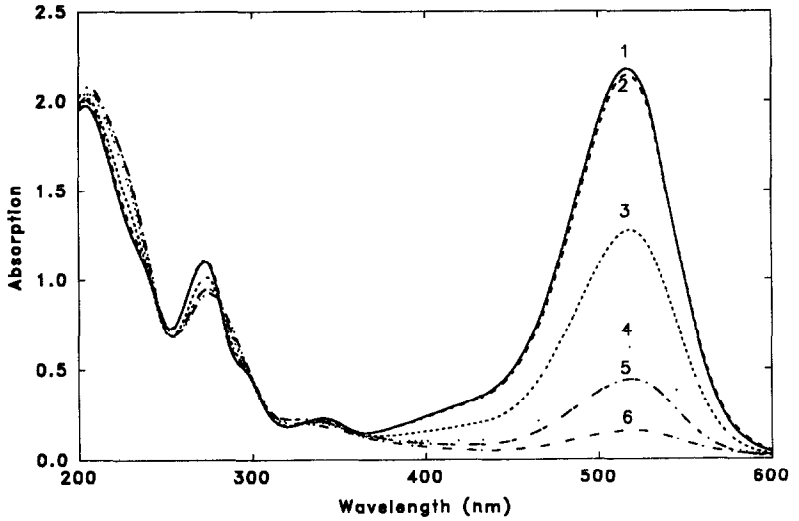

Fig. 6. Absorption spectra of malvidin 3,5-diglucoside in aqueous solution at several pH values ($T = 298$ K). (1) pH = 0; (2) pH = 0.9; (3) pH = 1.7; (4) pH = 2.2; (5) pH = 2.5; (6) pH = 2.8.

Table 2. Equilibrium constants at 25° and changes in the enthalpy and entropy of isomerization reactions

Reaction	K	ΔH^0 (kcal mol^{-1})	ΔS^0 (cal mol^{-1}K^{-1})
Flavylium↔hemiacetal 1	0.004_3	-0.8 ± 0.6	-12.6 ± 0.1
Flavylium↔hemiacetal 2	0.003_8	-0.1 ± 0.8	-10.4 ± 0.2
Hemiacetal 1↔*cis*-chalcone	0.40	4.4 ± 0.3	13.1 ± 0.1
Hemiacetal 2↔*cis*-chalcone	0.45	3.7 ± 0.2	10.9 ± 0.1
cis-Chalcone↔*trans*-chalcone	0.56	1.4 ± 0.3	3.7 ± 0.1

Quantum mechanical calculations

Quantum mechanical calculations were performed using AM1 and CNDO/CI/S methods (Quantum Chemistry Program Exchange, Indiana University). The calculated value of the enthalpy energy change for the *cis–trans* chalcone isomerization reaction ($\Delta H^0 = 1.76$ kcal mol^{-1}) is in very good agreement with the values obtained from the NMR measurements (Table 2). The positive value of ΔH^0 for the *cis–trans* isomerization indicates that the sterically most hindered isomer (*cis*) is also the most stable. This could be due to intramolecular hydrogen bonding between the ketone oxygen and the hydroxyl proton in the A ring. The flavylium–hemiacetal reactions are nearly athermic but entropically unfavoured due to entropy contribution of proton solvation. On the other hand, the ring opening (hemiacetal–*cis*-chalcone reaction) is moderately endothermic but is strongly entropically assisted, as expected.

EXPERIMENTAL

Sample preparation. Malvidin 3,5-diglucoside was purchased from Extrasynthese and used without further purification. The compound was freeze-dried once from D_2O and dissolved in DCl (approx. 0.1 M) to a final concn of 0.8–1.0 mM; concns were determined using $\varepsilon = 26500$ for the extinction coefficient of the flavylium form at wavelength 520 nm; the pH* of the starting soln was typically 0.85 and, when required, was changed by addition of small aliquots of 1 M NaOD or DCl. Quoted pH values are direct meter readings without correction for the isotope effect [9] and are denoted with an asterisk. Samples were analysed by NMR after allowing equilibration for at least 6 hr.

NMR spectroscopy. NMR spectra were obtained in either Bruker AMX-500 or Bruker AMX-300 spectrometers operating at 500.13 and 300.13 MHz, respectively. One-dimensional spectra were run with presaturation of the residual HDO resonance for 2.5 sec. The following conditions were used: 60° flip angle, 5.8 sec total recycle time and 32 K acquisition data points. Spectra were processed with 1 Hz line-broadening prior to Fourier transformation. Chemical shifts are referenced to internal sodium 3-(trimethylsilyl)-propanesulphonic acid sodium salt and sodium acetate was added as an int. standard for measurements of peak intensities. Two-dimensional exchange correlation spectra were run in the phase-sensitive mode according to ref. [10] and were acquired over a 3300 Hz bandwidth, collecting 2048 $(t_2) \times 512$ (t_1) data points and transformed to produce real (absorption mode) matrices consisting of 1024×1024 real data points. Mixing times in the range 0.5–2 sec were used.

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