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Behaviour of xyloisosaccharinic acid and xyloisosaccharino-1,4-lactone in aqueous solutions at varying pHs

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ABSTRACT

Xyloisosaccharinic acid is one of the major degradation products formed during the alkali catalysed hydrolysis of hemicelluloses. In acidic solution xyloisosaccharinic acid undergoes an acid catalysed lactonisation to generate xyloisosaccharino-1,4-lactone. We report here the solution phase properties of xyloisosaccharinic including measurement of its aqueous pK_a (3.00 ± 0.05) using ^{13}C NMR methods. We also report rate constants for the acid catalysed lactonisation, $k_{lact(D2O)}$, of xyloisosaccharinic acid and the results of our investigations of the kinetics of hydrolysis of xyloisosaccharino-1,4-lactone at acidic and basic pHs. The second-order rate constants for the hydrolysis reactions k_{HO-} (25 M^{-1} s^{-1}) and k_{D+} (4.13 E-4 M^{-1} s^{-1}).

1. Introduction

Saccharinic acids are a group of polyhydroxylated monocarboxylic acids and are the main decomposition products generated when polysaccharides and other carbohydrate materials are treated with aqueous alkaline solution.[1,2] They are formed in especially high amounts in alkaline wood pulping processes.[3] Saccharinic acids are of interest because they are potentially useful raw materials; they are good metal chelating agents[4–11] and, in their enantiomerically pure form, they are valuable carbon skeletons with predefined stereochemistry that can be easily functionalised for use in synthesis.[12–16] Saccharinic acids also have the potential to leach metal ions from cellulosic waste materials; this is of particular concern for the design of the deep-underground repositories currently under consideration[17–20] for the disposal of intermediate level radioactive waste. It is suspected that the production of saccharinic acids in the environment of a nuclear waste repository could influence the containment of radionuclides. Despite their importance, there are still gaps in our knowledge of the physical properties of many important saccharinic acids. Whilst a number of workers have explored the properties of isomeric glucoisosaccharinic acids (GISAH)[21–30] very little work has been undertaken looking at the properties of xyloisosaccharinic acid (**XISAH**, 3-deoxy-2-C-hydroxymethyl-D,L-tetronic acid). Whistler and Corbett[31] and Aspinall et al.[32] simultaneously demonstrated that XISAH is produced when small xylooligosaccharides are reacted with aqueous alkali. It has also been shown that significant amounts of XISAH are generated during alkaline treatment of corn[33,34] and birch[33,35] xylans. The alkali catalysed degradation of xylans is also responsible for the dominant production of XISAH during the alkaline pulping of hardwoods.[35,36] Like the corresponding GISAH, in acidic solution XISAH lactonises to give the corresponding xyloisosaccharino-1,4-lactone (**XISAL**, 3-deoxy-2-C-hydroxymethyl-D,L-tetrono-1,4-lactone, Scheme 1). Alén and Valkonen have crystallised XISAL and published its X-ray structure.[37]

Despite its potential value, little is known about the solution phase physical properties of XISAH. We report here the solution phase properties of XISAH including measurement of the aqueous pK_a of XISAH using ^{13}C NMR methods. We also report rate constants for the acid catalysed lactonisation of XISAH and the results of our investigations of the kinetics of hydrolysis of XISAL at both acidic and basic pHs.

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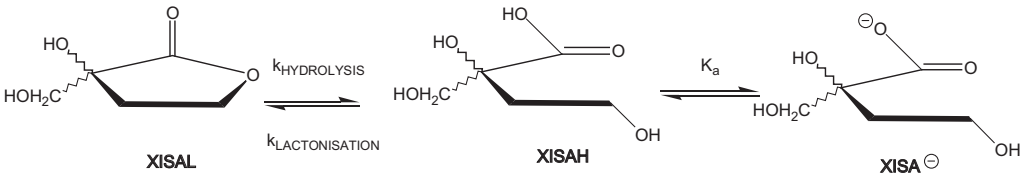


Scheme 1.

2. Experimental

2.1. Materials and chemicals

Xyloisosaccharino-1,4-lactone (XISAL, 3-deoxy-2-C-hydroxymethyl-D,L-tetrono-1,4-lactone) was prepared by GalChimia (Santiago de Compostela, Spain) using the procedures described by Aspinall et al.[32] The purity of the XISAH was determined by conversion to its pertrimethylsilyl-derivative and analysis by GC–MS. The product was trimethylsilylated with a mixture (1:1) of BSTFA and TMCS and was analysed with an Agilent 6890 Series GC System, equipped with an Agilent 5973 Mass Selective Detector and a Phenomenex ZB-5HT Inferno capillary column (30 m × 0.25 mm, film 0.25 μm). The temperature programme was 3 min at 200 °C, followed by 8 °C/min to 320 °C (for 15 min). The results indicated *c* 99% purity of XISAL, and the mass spectrum was in good agreement with the literature data.[38] MS (*m*/*z*, rel intensity): 261 [10 (M-15)], 246 (100), 233 (8), 190 (10), 147 (77), 143 (35), 103 (27), 73 (77).

All other reagents were analytical grade reagents and were purchased from the Aldrich Chemical Company (Poole, UK).

2.2. Determination of the aqueous pK_a of XISAH

XISAL (20 mg, 0.151 mmol) was dissolved in D_2O (1 mL) and the pH of the solution was varied between pD 1 and 11 using either DNO_3 or NaOD and reporting pD as the measured pH +0.4. Samples were quickly transferred to NMR tubes and spectra were recorded at a probe temperature of 300 K on a Bruker Avance 500 MHz (125 MHz ^{13}C) spectrometer. Proton decoupled and NOE enhanced carbon spectra were recorded using 256 scans employing a wait time between scans of 2 s for experiments where chemical shifts were being measured and 10 s for those spectra where carbon signals were being integrated; ten seconds was chosen to allow complete relaxation (>5 × T_1) of the carbon resonances used in the kinetic studies. A capillary insert, containing $CDCl_3$, was used as an internal reference. For the assignment of resonances to individual carbons in XISAH and XISAL a series of 1D and 2D-NMR spectra were recorded at both high and low pHs and samples were allowed to fully equilibrate before spectra were recorded, that is, until single sets of resonances were observed, at extremes of pH, less than 30 mins was needed for complete transformation to either XISAL or XISA. Spectra recorded included proton, Carbon DEPT, COSY, HMBC and HSQC and these were acquired using Bruker standard pulse sequences. The solution pH was measured in the NMR tube using a Beckman ø40 pH Meter (High Wycombe, UK) in combination with a Hanna instruments glass long reach NMR pH electrode (Mannheim, Germany). The pK_a of the acid was determined using the Henderson–Hasselbach equation: pK_a = pD +log{[AD]/[A−]}.[39,40]

2.3. Following the kinetics of the hydrolysis of XISAL in alkaline solution

A stock solution of XISAL (20 mg/100 μL HCl (0.1 M)) was prepared prior to experiments. Kinetic runs were initiated by adding the XISAL (50 μL) stock solution to ultrapure water (65 mL) in a round bottom flask and the solution was stirred under an atmosphere of nitrogen, The reaction vessel was attached to a Metrohm autotitrator (Titrano model 857, Metrohm, Runcorn, UK) and was maintained at 25 °C throughout the experiments. Fresh sodium hydroxide solutions were used as the titrant, these were prepared from a volumetric standard prior to the reaction and the titrant was flushed with nitrogen to avoid absorption of carbon dioxide. The appropriate start pH was selected and the volume of titrant added as a function of time was recorded. All pH measurements were recorded in-situ using a Unitrode combined glass pH electrode equipped with a built-in PT1000 temperature sensor (Metrohm, Runcorn, UK).

2.4. Following the inter-conversion of XISAH and XISAL in acidic solution

The kinetics for the lactonisation of XISAH were followed using NMR spectroscopy. Samples of XISAL were dissolved in D_2O (20 mg/mL) and the pH of the solution was initially increased to 12 and the sample was left for ten minutes to ensure complete conversion of the XISAL to the anion XISA. The sample was cooled in an ice bath before adjusting the pH of the solution to the required pD (1–2.5) through the addition of DNO_3. Samples were immediately transferred to the NMR spectrometer and spectra were recorded at regular periods over a period of several days or until no further reaction was observed. When spectra were not being recorded samples were stored at 25 °C; the pD of the reaction solution was monitored throughout the course of the reaction.

An attempt was made to monitor the equilibrium between the lactone and the free acid using NMR spectroscopy: samples of the lactone were dissolved in D_2O (10 mg/mL) and the pD of the solution was reduced to acidic pDs through the addition of DNO_3. A series of solutions of varying pD (1–5) were prepared and these were stored, under an atmosphere of nitrogen, at 25 °C for a period of up to two months.

2.5. Analysis of the mechanism of the base catalysed sovolysis reactions

For the hydrolysis reactions using oxygen-18 labelled water, XISAL (10 mg) was dissolved in a solution of ^{18}O-labelled H_2O (98% 1 mL) and the pH of the solution was maintained at 7 for ten days (0.1 M NaOH was added as required). After ten days the sample was diluted with ultra-pure water before analysis by LC–MS–MS using an Agilent 6460 triple quadrupole mass spectrometer (Cheadle, UK) fitted with a JetStream electrospray source interfaced with an Agilent 1290 HPLC. Samples (5 μl) were separated on a Zorbax Extend C-18 column (1.8 μm particle size and 2.1 × 50 mm) using a mobile phase containing acetonitrile (A) and aqueous ammonium acetate (20 mM, B) operating with isocratic elution (90% A:10% B) with a flow rate of 0.25 mL/min and at a column temperature of 25 °C. The electrospray interface was operated using negative ion polarity. The JetStream source gas temperature was maintained at 300 °C, with a gas flow of 10 L/min and a nebuliser gas pressure of 15 psi. An optimal spray was obtained with the capillary voltage and the nozzle voltage set at 3500 and 500 volts,

respectively, and employing a delta EMV of 150 volts. The sheaf gas temperature was maintained at 250 °C and a flow rate of 7 L/min. The mass spectrometer was operated using product ion scanning, for the parent ion the fragmentation voltage was set at 100 volts and collision-induced dissociation was monitored over a range of collision energies between 5 and 20 volts. For the pseudo-MS–MS the fragmentation voltage was increased to 150 volts in order to favour production of the required daughter ions.

3. Results and discussion

Xyloisosaccharinic acid (XISAH) is a small polyhydroxylated aliphatic carboxylic acid and, in aqueous solution, will exist as the free acid (XISAH) at moderately acidic pHs and as its conjugate base (XISA) at pHs significantly above the acid's pK_a. In common with other 4-hydroxy-substituted monocarboxylic acids, XISAH is known to cyclise at low pHs to form xyloisosaccharino-1,4-lactone (XISAL).[32] In order to determine the relative proportions of the three species present, a study of the variation of the position and number of the ^{13}C NMR resonances of a solution of XISAH was undertaken at various pHs.

At neutral and alkaline pHs the ^{13}C spectrum recorded for XISAH contains five resonances corresponding to the five carbon atoms of the anion (XISA, bottom trace Fig. 1). The identity of the individual signals was determined by analysis of both the carbon spectrum and the carbon DEPT-135 spectra (not shown): the three methylene groups gave negative signals on the DEPT-135 spectrum and the signal at lowest field was assigned to C3 (36.7 ppm) the other two methylene resonances are shifted to higher field by the presence of the electron withdrawing hydroxyl groups. The two carbon signals missing from the DEPT-135 spectrum were assigned to the quaternary centres of XISA, that is, C1 & C2, the lowest field signal being assigned to the carbonyl carbon (C1,179.9 ppm) and the second signal to C2 (78.3 ppm). Analysis of a combination of the COSY and HSQC spectra allowed the assignment of the remaining two methylene groups: the C4 methylene gives rise to a signal at 57.8 whilst the C5 methylene gives rise to the resonance centred at 66.6 ppm.

When a solution of XISAH was acidified to a pH less than 3, the initial spectrum contains the same five resonances, although some have moved significantly (see discussion below) and this is consistent with protonation of the anion and formation of the free acid XISAH in solution. However, a further five peaks slowly appeared over time and these additional peaks were attributed to being those of the lactone (XISAL, top trace Fig. 1). A similar set of DEPT-135 and 2D-COSY, HMBC and HSQC spectra were used to confirm the location of the individual resonance lines of the carbons in the lactone: C1 (180.4 ppm) C2 (75.9 ppm) C3 (32.7 ppm) C4 (67.2 ppm) and C5 (64.3 ppm). The down-field shift of C4 matches a similar shift observed for C4 of alpha-GISA[25] on lactonisation and is consistent with acylation of the primary alcohol and formation of a five member-ring lactone.

In order to determine the aqueous pK_a of the carboxylic acid of XISAH, the variation in the chemical shifts of the carbons signals of XISA/H with the pD of the solution was monitored. The chemical shift for three of the five carbons varied and a sigmoidal dependence of the chemical shift of C1, C2 and C4 on the pD of the solution was observed (Fig 2a–c).

As was expected, the largest shift was observed for the carbonyl carbon (C1 $\Delta\delta = 2.22$ ppm) identifying that the sigmoidal dependence is related to protonation of the carboxylate anion. The upfield shift of the carbonyl carbon signal is similar to that observed

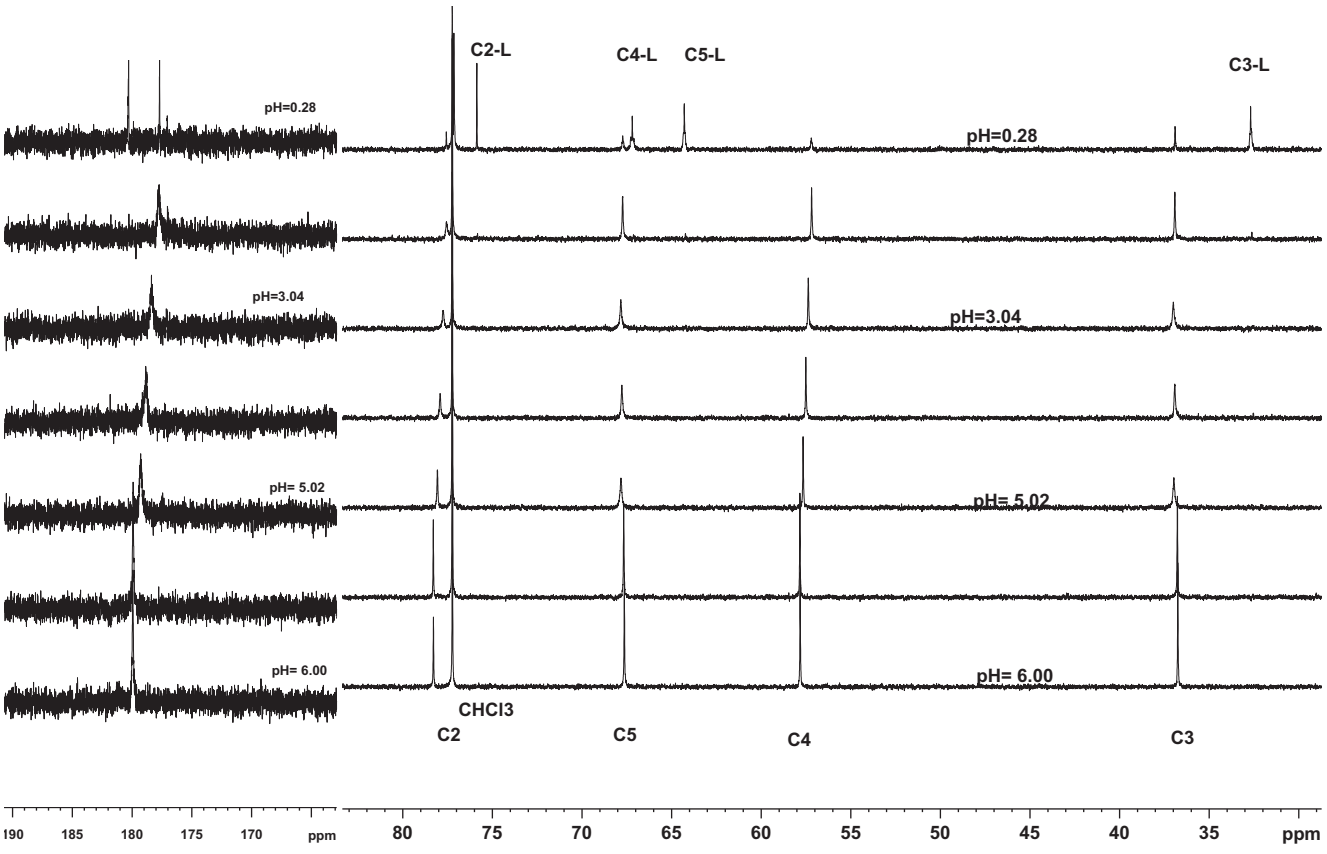


Figure 1. ^{13}C chemical shifts (δ in ppm) for the resonances of XISA/H (for assignment of the signals in XISA see the bottom spectrum) and XISAL with residual XISAH (for assignment of the carbon signals in the lactone-see the top spectrum) at various pDs recorded at 300 K.

for protonation of the carboxylate group in other saccharinic acids.[25,41] As proton transfer between the free acid and anion will be rapid, the observed chemical shift δ(pD) for each carbon signal will reflect the mole fraction of each species present and can be fitted to the equation:

$$\delta(\mathrm{pD}) = \delta_{\mathrm{acid}} + \delta_{\mathrm{base}}[10^{-\mathrm{pD}}/(K_a + 10^{-\mathrm{pD}})]$$

The measured pK_a's from the individual resonances are 3.37 (C1) 3.47 (C2) and 3.36 (C4) with the mean value being 3.40 ± 0.05. Glasoe and Long[42] established a ΔpK_a value of −0.4 for transfer from D_2O to H_2O for a range of weak acids and therefore the corresponding pK_a in H_2O can be estimated as 3.0. The measured pK_a is slightly lower than that measured for the two diastereoisomers of GISAH recorded using NMR chemical shifts (alpha-GISAH = 3.36[25] and beta-GISAH = 3.61[41]) and is lower than the value of 3.49 calculated by Käkölä et al. for XISAH.[43] Given that the substituents immediately adjacent to the carboxylate group are the same in XISAH and GISAH it is difficult to account for the increased acidity of XISAH. However, there is some evidence for the presence of a hydrogen-bonding interaction between the carboxylate group and the hydroxyl substituent at C4. The sigmoidal dependence of the C4-chemical shift of XISAH as a function of pD and the significant movement in the resonance position of the C4 carbon on protonation of the carboxylate group suggests that there is direct interaction between the substituents at these two centres. Cho et al.[25] suggested that for alpha-GISAH a hydrogen bond exists between the carboxylate group and the secondary alcohol at C4, a similar hydrogen bond in XISAH could account for the movement in the chemical shift for C4 observed in the present study. Differences in the extent of this hydrogen-bonding, which involves a primary hydroxyl group in XISAH and a secondary hydroxyl group in GISAH, could potentially account for the observed difference in the measured pK_a values.

Our NMR studies indicated that at low pHs XISAH lactonises to form XISAL and we were interested in measuring the rate constants for the inter-conversion of the lactone (XISAL) and the free acid (XISAH). When XISAL was dissolved in D_2O at neutral pH's (6–8) the NMR spectrum that was initially recorded included large signals for the lactone, the intensity of these signals slowly reduced over-time and the drop in signal intensity was accompanied by a corresponding drop in the pH of the sample, indicating that lactone hydrolysis was occurring. In order to monitor the kinetics of the hydrolysis reaction, samples of XISAL were hydrolysed at fixed pHs (7–10.5) in an autotitrator and the consumption of base was followed over time. At a fixed pH, the volume of base consumed in the hydrolysis reactions decreased exponentially with time indicating a first-order reaction and a plot of the logarithm of the volume of base added against time was linear from which it was possible to calculate a pseudo-first order rate constant (k_{obs}) for the hydrolysis at that pH. This process was repeated for a range of hydroxide concentrations and a pH rate profile for the base catalysed hydrolysis reaction was constructed (Fig 3). From inspection of the pH rate profile it is clear that at the lowest pHs studied the hydrolysis of the lactone is extremely slow ($t_{1/2}$ pH 7 of 26 h) whilst at high pHs the hydrolysis reaction is very fast ($t_{1/2}$ pH 10.5 of 90 s). The reactions between pH 8.5 and 10.5 were allowed to run to completion ($5 \times t_{1/2}$) during which period one equivalent of base was consumed indicating that above the pK_a of the acid the lactone (XISAL) does not exist in equilibrium with the anion (XISA) and that if a solution of the lactone is left for long-enough it will undergo complete hydrolysis. At high pHs the rate profile has a slope of one and this is consistent with a bimolecular base catalysed reaction. The vast majority of simple esters undergo base catalysed hydrolysis via a $B_{AC}2$ mechanism, that is, through nucleophilic attack at the carbonyl carbon, however, at neutral pHs a number of substituted cyclic lactones including β-butyrolactone[44] are able to react via attack of the nucleophile at the alkoxy-carbon with accompanying cleavage of the alkoxy-oxygen bond, that is, via a $B_{AL}2$ mechanism. The $B_{AL}2$ mechanism normally only operates when attack of the nucleophile at the acyl-carbon is blocked by bulky substituents located adjacent to the carbonyl group. In the X-ray structure there is evidence for crowding of the carbonyl centre by the adjacent C2 hydroxyl and hydroxymethylene substituents. In order to get information about the position of attack of the nucleophile in XISAL, the hydrolysis reaction was performed at pH 7 in ^{18}O-labelled water and the location of the label in the product was identified by LC–MS–MS. Mass spectra obtained for the reactions performed in ^{16}O and ^{18}O labelled water (Fig 4) show the expected molecular ions for XISA at m/z of 151 Da for the ^{18}O labelled nucleophile and at m/z of 149 for the ^{16}O labelled nucleophile. The highest mass fragment in both systems arises from the expulsion of the C2-hydroxymethylene group, as formaldehyde, via a McLafferty-type rearrangement (Scheme 2). A similar rearrangement has been reported for 2,3-dihydroxypropanoic acid which possesses the same β-hydroxycarbonyl group that is present in XISA.[45] The McLafferty rearrangement initially generates a dihydroxyenolate which will tautomerise to form the 2,4-dihyroxybutanoate anion which is visible at m/z = 121(^{18}O)

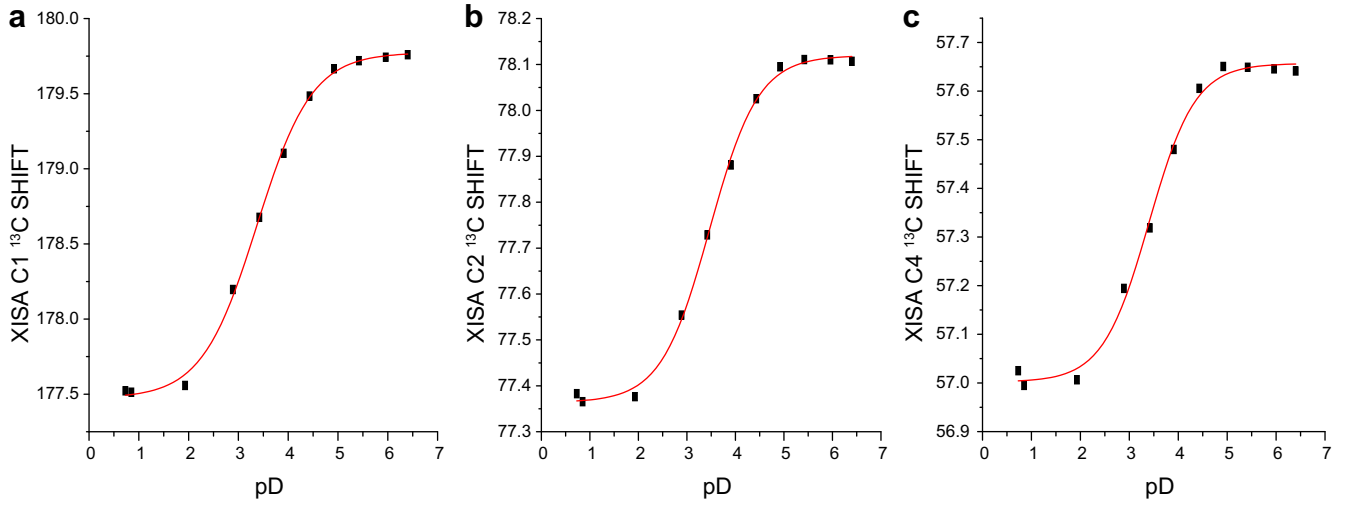


Figure 2. Variation of the ^{13}C chemical shifts of XISAH as a function of pD. Solid line represents the fit to the equation: $\delta(\mathrm{pD}) = \delta_{\mathrm{acid}} + \delta_{\mathrm{base}}[10^{-\mathrm{pD}}/(K_a + 10^{-\mathrm{pD}})]$ giving a mean pK_a in D_2O of 3.40 ± 0.05.

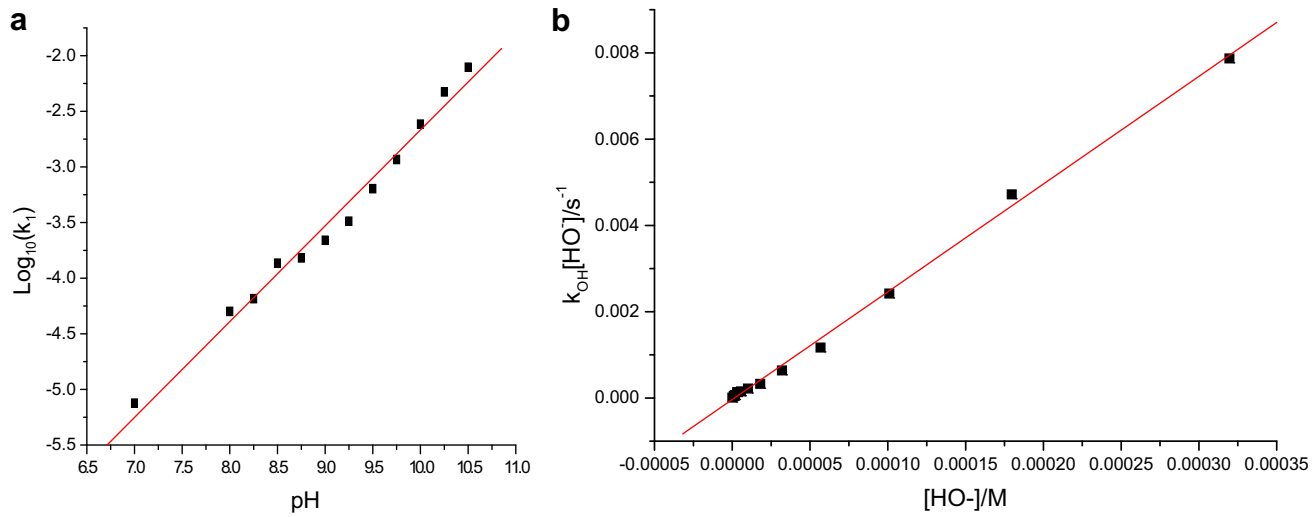


Figure 3. Left-hand side: pH versus rate profile for the hydrolysis of XISAL at 25 °C. Right-hand side: plot of k_{obs} versus [HO^-] for the hydroxide catalysed hydrolysis of XISAL.

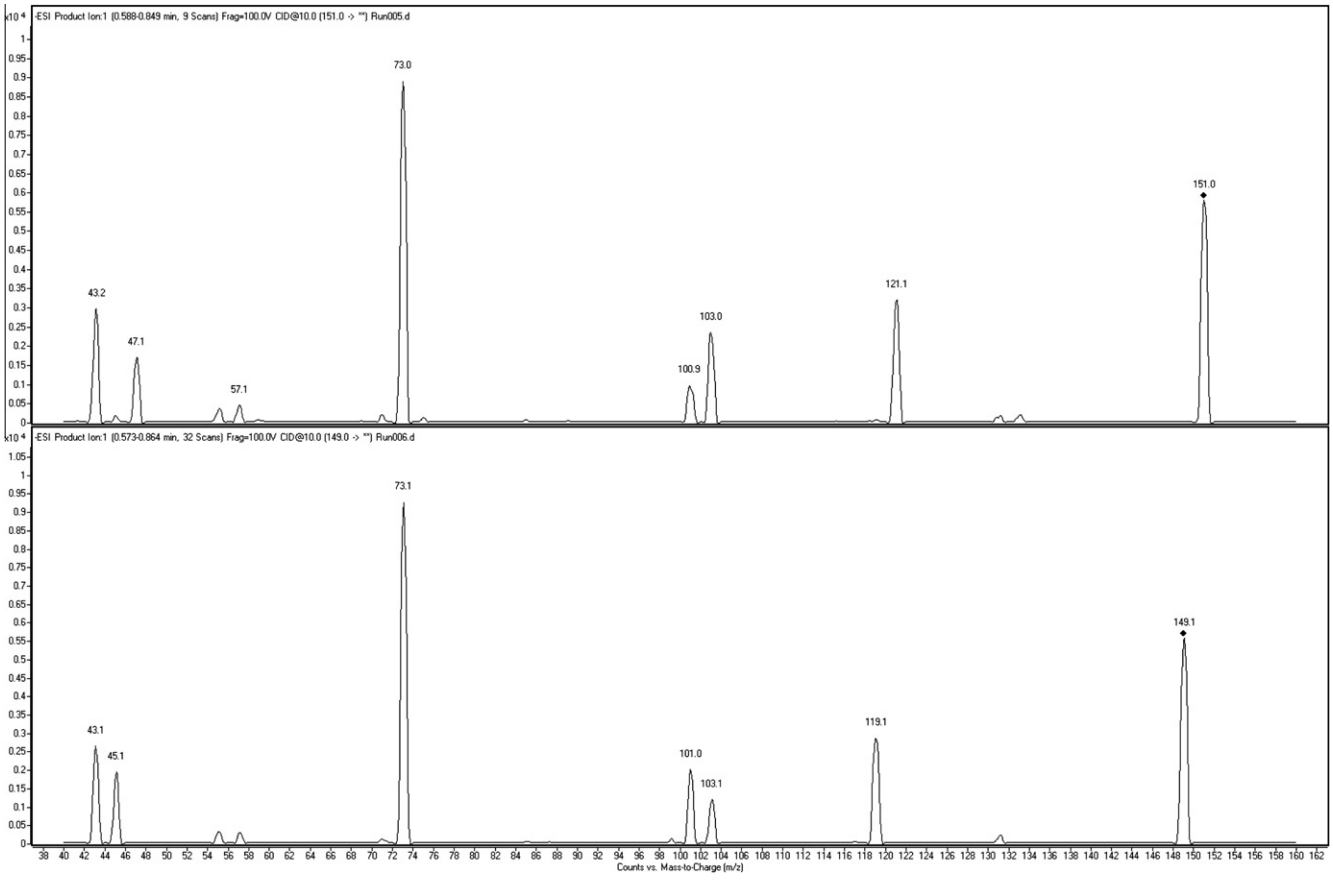
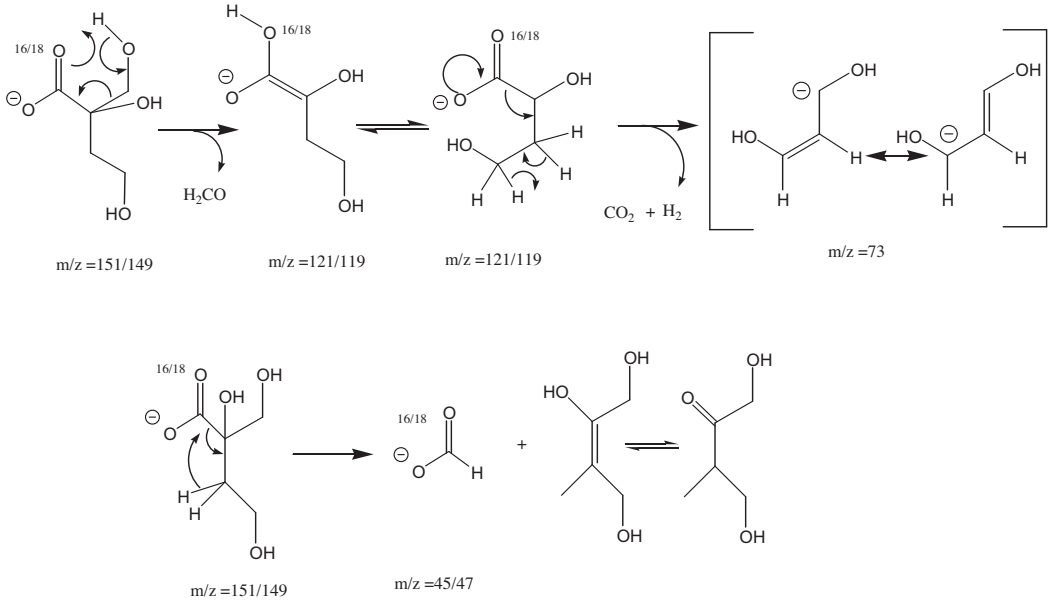


Figure 4. Top, mass spectrum for an XISA sample prepared by reaction of XISAL with H_2 ^{18}O/NaOD which was maintained at pH 7 and at room temperature for 12 days and employing a collision energy of 10 volts. Bottom, mass spectrum for an XISA sample prepared by reaction of XISAL with H_2 ^{16}O/NaOD which was maintained at pH 7 and at room temperature for 12 days and employing a collision energy of 10 volts. Further details of how the spectra were recorded are listed in the Section 2.

and 119 (^{16}O). The next highest mass fragments, m/z = 103 (^{18}O) and 101 (^{16}O), can be attributed to the loss of water from 2,4-dihydroxybutanoic acid and this was confirmed by studying the daughter ions generated by collision-induced dissociation (CID) of the m/z 121 and 119 ions. The third highest mass fragment is the most abundant fragment and in both systems this fragment occurs at m/z of 73, signifying that the isotopic label has been lost as part of a neutral molecule, this is likely to arise from the elimination of the elements of formic acid in the concomitant loss of carbon dioxide and molecular hydrogen from 2,4-dihyroxybutanoate. That the m/z of 73 is generated from both the 119 and 121 ions was confirmed when it was produced as a daughter during CID of the m/z 121 and 119 ions. A very similar fragmentation has previously been reported by Bowie and co-workers[46] in the spectra obtained



Scheme 2.

for CID of carboxylate ions that are not able to form stabilised carbanions. The loss of CO_2 and H_2, and the accompanying loss of mass of 48 and 46 in the two systems, clearly identify that the label is present in the carboxylate group and this infers that lactone hydrolyses occur via the $B_{AC}2$ mechanism. A second piece of evidence that supports the location of the label in the carboxylate group is the observation of fragments at *m/z* 47 (^{18}O) and 45 (^{16}O) as abundant fragments of the molecular ion but only as minor ions in the spectrum formed from the CID of *m/z* 121 and 119. The two fragments are likely to be derived from the elimination of formate anion from XISA (Scheme 2).

A measure of the relative reactivity of XISAL can be obtained by determining the second-order rate constant for hydrolysis with those measured for other lactones. Using a value of 1.01×10^{-14} for the ion product of water, K_w, the second order rate constant for the hydroxide catalysed hydrolysis of XISAL can be determined from the slope of a plot of hydroxide concentration against k_{obs} ($k_{obs} = k_{HO-}[HO^-]$, Fig 3b). The value obtained $k_{HO-} = 25\ M^{-1}\ s^{-1}$ is approximately 25-fold lower than the value estimated for butyrolactone, $k_{HO-} = 1.0\ M^{-1}\ s^{-1}$, determined by extrapolation of data reported by Blackburn and Dodds[47] measured at 46 °C ($k_{HO-} = 4.03\ M^{-1}\ s^{-1}$). One possible explanation for the increased reactivity of XISAL is that the adjacent electron withdrawing hydroxyl group activates the carbonyl carbon to nucleophilic attack.

In acidic solution, XISAL can exist in equilibrium with XISAH. Normally, this would offer the opportunity to study the equilibration process and allow us to determine the relative abundance of the lactone and free acid forms present at different pHs, similar studies have been performed on GISAH/L.[48] In a first attempt to monitor the relative proportions of the two species XISAH and XISAL present, a number of experiments were performed in which a solution of the XISAL was prepared in D_2O at acidic pDs, between 0 and 5, and these were left in the dark under an atmosphere of nitrogen for a period of up to two months and NMR spectra were recorded periodically through-out the course of the reaction. Unfortunately, at the higher pDs, 4 and 5, the time taken to reach equilibrium was too long and the free acid was only present in very small amounts. In order to overcome the small extent of reaction, a second set of experiments was performed but starting in the reverse direction, that is, adding the XISAH to acidic solution and monitoring the loss of XISAH and the production of XISAL at a fixed temperature of 25 °C. These experiments were followed by monitoring the ratio of XISAH:XISAL using ^{13}C NMR (Fig 5). The XISAH concentration decayed exponentially with time allowing the half-life for the equilibration reaction to be determined (pH 1.1 $t_{1/2} = 4.75$ h and at pH 1.97 $t_{1/2} = 48.2$ h). Assuming both the forward and reverse reactions are acid catalysed, this would suggest that the half-life for the equilibration process would be approximately 500 and 5000 h at pHs 3 and 4, respectively. Given the slow rates of reaction, the time for complete equilibration at pH 3, that is, $5 \times t_{1/2}$, would be approximately three months and this figure is consistent with the very slow conversions observed in the first set of experiments.

For the reactions performed at pDs between 1 and 2, the observed rate constant (k_{obs}) measured for equilibration at a fixed pD is the sum of the forward ($k_{lact/D2O}$)[D+] and reverse rate constants ($k_{hyd/D2O}$)[D+] and given that the equilibrium concentration ($K_{equ/D2O}$) is equal to the forward rate constant ($k_{lact/D2O}$) divided by the reverse rate constant ($k_{hyd/D2O}$) and is equal to the relative proportions of XISAH/L present at the end of the reaction, it is possible

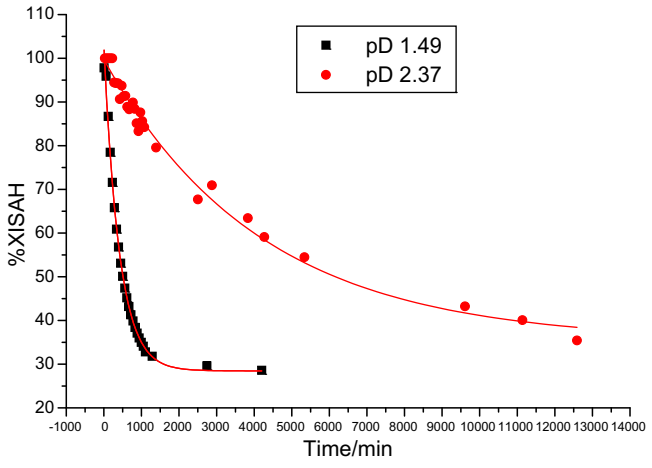


Figure 5. Plot of the % XISAH remaining in acidic solution (pD 1–2.5) as a function of time, the % values were calculated from the integration of the carbon signals recorded at 300 K and using extended delays between pulses for quantification (see Section 2).

Table 1
Observed rate constants (k_{obs}/s-1) and equilibrium constant ($K_{equ/D2O}$) measured for the lactonisation of XISAH at various values for pD determined by following the exponential decay of the XISAH as a function of time using NMR at 25 °C. The second-order rate constants for the acid catalysed lactone hydrolysis ($k_{hyd(D2O)}[D^+]$/s-1) and $k_{lact(D2O)}[D^+]$/s-1 were calculated from $k_{obs}[D^+] = k_{hyd(D2O)}[D^+] + k_{lact(D2O)}[D^+]$ and $K_{equ/D2O} = k_{lact(D2O)}/k_{hyd(D2O)}$)

pD	k_{obs}/s-1	$K_{equ/D2O}$	$k_{hyd(D2O)}[D^+]$/s-1	$k_{lact(D2O)}[D^+]$/s-1
1.49	6.73 E-5	5.13	1.09 E-5	5.63 E-5
1.67	5.17 E-5	3.22	1.23 E-5	3.94 E-5
2.37	3.48 E-6	2.21	1.41 E-6	2.40 E-6

to determine individual rate constants for the acid catalysed lactone hydrolysis and lactone formation reaction and the value for $K_{equ/D2O}$ at that pH. The values calculated for $k_{hyd/D2O}$, $k_{lact/D2O}$ and $K_{equ/D2O}$ at acidic pHs are presented in the Table 1.

Ekberg et al.[48] have measured the same data set for the interconversion of GISAL and GISAH at pH 1 (K_{equ} (6.60) $k_{hyd/H2O}$ = 1.2 E-5 s^{-1} and $k_{lact/H2O}$ = 8.0 E-5 s^{-1}) however, caution should be practised in attempting a direct comparison of these figures because of the different lactone structures and the requirement to consider solvent isotope effects. Given the likely differences, it is surprising that the numbers are similar. As stated above, the acid catalysed hydrolysis reactions were very slow and when combined with the limited availability of XISAH this meant that we were only able to follow the reaction over a very limited pD range, that is, between 1 and 2.5. However, using the data presented in Table 1, we can get an estimate for the second order rate constant for the acid catalysed hydrolysis reaction k_{D+} as 4.13 E-4 M^{-1} s^{-1}.

4. Conclusions

The pK_a of XISAH has been measured using NMR methods and the value (3.0 ± 0.05) is lower than that measured for both alpha and beta-GISAH using the same procedures. The rate constants for hydrolysis of the lactone XISAL have been measured in both acidic and basic solutions with the half-lives for reaction in acidic and neutral solution being large. These slow transformation rates will need to be considered when calculating the relative ratios of the two species XISAH/L present in solution and this will be an important consideration in calculating solubility and complexation data for XISAH: between pH 4 and 7 an aqueous solution will take several months to achieve complete equilibration.

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