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HIGH FIELD ^{1}H N.M.R. STUDIES ON THE AMMONIATION OF AFLATOXIN B_1

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Summary: The use of high field ^{1}H n.m.r. to follow the reaction of aflatoxin B_1 with ammonium hydroxide has been investigated. The time course of the formation of aflatoxin D_1 and MW206 has been established and a mechanism is proposed for the parallel formation of the two major breakdown products.

Since the well publicised incident in England, in 1960, when 100,000 turkey poults were affected by the previously unknown "Turkey-X disease",[1] and the subsequent isolation and characterisation of aflatoxin B_1 (1) as the major mycotoxic contaminant of Brazilian groundnut meal fed to the fowls, there has been considerable interest in ensuring that such cases of toxicosis do not recur. The danger of mycotoxins to man, whether directly through contaminated foods, or indirectly, from the products of animals ingesting mouldy foodstuffs, is such that all necesary steps should be taken to eliminate that risk. While it is sensible to store foodstuffs under conditions of temperature and humidity that minimise fungal growth, it is often the case that the product has been spoiled before harvest, and already contains considerable amounts of mycotoxin. The detection of mycotoxins is therefore very important, and intensive research has resulted in sensitive methods of quantifying aflatoxins in a wide range of commodities, leading to the setting of a 20 ppb limit by the US Food and Drug Administration.

Once a commodity has been identified as being contaminated beyond a level fit for human or animal consumption, the problem arises of what to do with it. The removal of mouldy peanuts for example, by electronic sorting methods, is an attractive idea, but the fact that aflatoxins diffuse away from the mycelium means that residual contamination will remain. Alternatively, aflatoxin contaminated peanuts may be diverted to the production of edible oil, since the process results in an aflatoxin-free product,[3] but the toxins remain in the residual meal, which is still unfit for use as feed. Reductions in mycotoxin levels are usually achieved during cooking processes, such as the dry roasting of peanuts, or the popping of corn. However, in the case of aflatoxins, the reduction is modest, and the consumer is unlikely to accept a product which has been contaminated at such a late stage. Therefore, a number of chemical methods for treatment of contaminated feeds have been investigated, and in general it has been found

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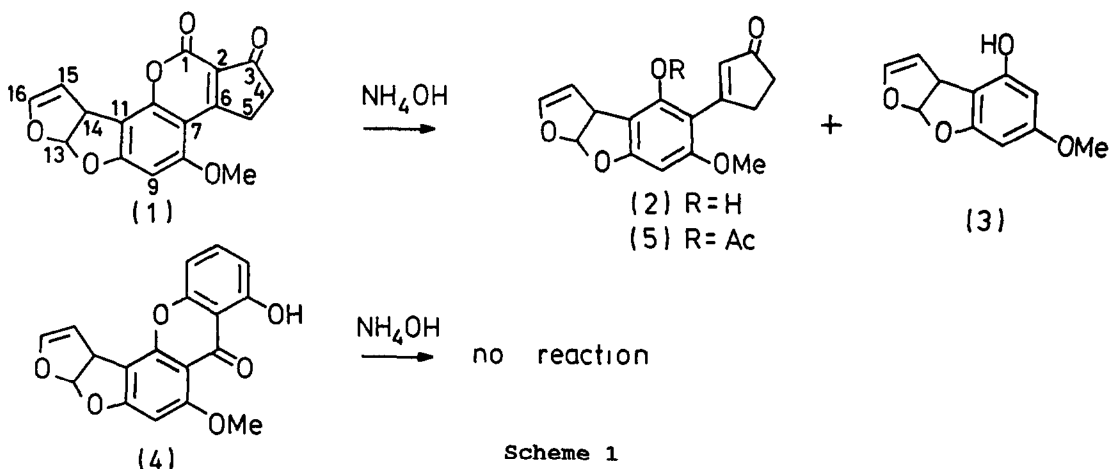
that those based on ammoniation have proved the most satisfactory both in reducing the level of contamination and in retaining the effective food value of the product.[4] Ammonia has been used to destroy aflatoxins in various feedstuffs either in its gaseous form, or as ammonium hydroxide solution, and at various temperatures, pressures, moisture content and reaction times.[4] Since showing promise as a means of detoxifying aflatoxin contaminated products, the ammoniation process has been investigated in order to determine the degradation products.

In a model reaction, Lee *et al* heated aflatoxin B_1 with concentrated ammonium hydroxide solution in a sealed Parr bomb at 100°C for one hour.[5] The major component, isolated by chromatography, was a non-fluorescent phenol of molecular weight 286, lacking the lactone carbonyl of aflatoxin B_1. The structure (2) based on spectroscopic and chemical analyses, was proposed and the compound named aflatoxin D_1, as it is formally derived from decarboxylation of the lactone ring opened form of aflatoxin B_1. In the same year, Kiermeir and Ruffer reported isolating the same compound from the degradation product mixture of aflatoxin B_1 in a diethanolamine buffer, and also from treatment with sodium hydroxide.[6] Aflatoxin D_1 has also been prepared in 28% yield by heating aflatoxin B_1 with concentrated ammonium hydroxide in a sealed flask at 50°C for 14 days.[7] Cucullu *et al* isolated another product from the reaction of aflatoxin B_1 and concentrated ammonium hydroxide.[8] This compound was another, less polar, non-fluorescent phenol of molecular weight 206, lacking the cyclopentenone ring of aflatoxin B_1 and structure (3) was assigned mainly on mass spectral evidence. This compound, previously prepared by Buchi[9] as a key intermediate in an aflatoxin B_1 synthesis, was also postulated as arising from a ring opened form of aflatoxin B_1. These transformations are summarised in Scheme 1.

The initial aim of the studies reported below were to isolate and identify the products resulting from treatment of aflatoxins with ammonium hydroxide and to study the mechanism of their formation. Ultimately the nature of the binding of the ammoniation products to meal matrices and the way in which meal matrices modify the degradation process need to be



Scheme 1

Table 1 ^{1}H N.m.r. spectra of aflatoxin B_1 (1), aflatoxin D_1 (2), MW206 (3) and aflatoxin D_1 acetate (5)

Hydrogen	(1)^a	(2)^b	(2)^c	(3)^a	(3)^c	(5)^a
2		6.48(t,2)	6.46(s)			6.15(t,2)
4	2.62(m)	2.52				2.46
5	3.38(m)	3.22				2.97
7				5.91(d,2)		
9	6.38(s)	6.20	5.67	6.11(d,2)		6.46
13	6.79(d,7)	6.73	6.47	6.67	6.43	6.69
14	4.74(dt,7,2)	4.67	4.33	4.54	4.30	4.47
15	5.45(dd,3,2)	5.43	5.31	5.32	5.28	5.13
16	6.40(dd,3,2)	6.50	6.28	6.43	6.26	6.45
OMe	3.93(s)	3.81	3.52	3.72	3.48	3.79
OH				4.89(br)		
OAc						2.22(s)

Chemical shifts in ppm for solutions in (a) $CDCl_3$, (b) $CDCl_3$-CD_3OD and (c) ND_4OD. All structures are numbered according to the equivalent positions in aflatoxin B_1. Coupling constants are in Hz; unless otherwise stated multiplicities and couplings are the same as in aflatoxin B_1

examined, but clearly a good understanding of the degradation of the aflatoxins themselves needs to be obtained at the outset. It seemed likely that the mechanism of ammoniolysis of aflatoxin B_1 could be studied using ^{1}H n.m.r. to follow the time course of the formation of the major degradation products and to observe any transient intermediates formd. Surprisingly the ^{1}H n.m.r. spectrum of aflatoxin D_1 has not been reported, although details of the ^{13}C n.m.r. spectrum are available.[10] The first task was to obtain samples of the major degradation products to provide suitable reference spectra for the n.m.r. studies.

Aflatoxin B_1 (1) was refluxed overnight in concentrated ammonium hydroxide solution. Analytical thin layer chromatography (tlc) of the product mixture showed the presence of aflatoxin B_1 and a more polar compound, corresponding to aflatoxin D_1 (2). When the closely related xanthone sterigmatocystin (4) was subjected to the same conditions, it was recovered unchanged, indicating that the bisdihydrofuran moiety, present in both sterigmatocystin and aflatoxin B_1, is stable to ammonium hydroxide.

In order to investigate prolonged heating with ammonium hydroxide, a closed system was used. Aflatoxin D_1, produced from the reaction of aflatoxin B_1 with concentrated ammonium hydroxide in a sealed flask, kept at 56°C for 14 days,[7] was found to be insufficiently soluble in deuterated chloroform, acetone, or dimethyl sulphoxide for n.m.r. analysis. therefore, the sample was treated with pyridine and acetic anhydride, to form the acetate (5), which was soluble in chloroform. The acetate was also isolated in 36% yield by acetylation of the crude mixture following treatment of aflatoxin B_1 with concentrated ammonium hydroxide, and subsequent preparative tlc. The proton n.m.r. (Table 1) of this compound was consistent with the proposed structure (5) as described below.

The presence of the dihydrofurobenzofuran moiety was evident from the coupling pattern. The doublet of triplets at 4.47 ppm, due to the benzylic

proton, was the result of a coupling of 7 Hz to the acetal proton at 6.69 ppm, and couplings of 2 Hz, to H-15 at 5.13 ppm, and allylic coupling to H-16, which was present as a doublet of doublets at 6.45 ppm. The presence of an olefinic proton as a triplet at 6.15 ppm which collapsed to a singlet on irradiation of the methylene multiplet at 2.97 ppm, and the concomitant simplification of the multiplet at 2.46 ppm into an AB coupling pattern, confirmed the presence of the cyclopentenone system; the methylenes at 2.46 and 2.96 ppm being α and β respectively to the carbonyl function. Further experimentation revealed that aflatoxin D_1 would dissolve in a mixture of deuteriochloroform and deuteriomethanol, giving a proton n.m.r. spectrum (Table 1) similar to that of the acetate (5).

The molecular weight 206 compound (MW206) (3) was also found to be present by analytical tlc of aflatoxin B_1 ammoniation mixtures, showing up as a characteristic orange spot following spraying with Fast Blue B.[7] However, since it was present only in small quantities, larger scale ammoniation of aflatoxin B_1 was carried out in a sealed flask at 50°C for 21 days. In addition to aflatoxin D_1 (57%) and aflatoxin B_1 (15%), MW206 was isolated from the product mixture by preparative tlc, in 9% yield. There was now sufficient material for proton n.m.r. spectroscopy, and the resulting spectrum (Table 1) was indistinguishable from that of the racemic compound, available by synthesis.[9] During these separations it was evident that there were no other significant products present in readily isolable quantities.

To determine whether or not n.m.r. studies would be feasible, a lyophilised sample of ammoniated aflatoxin B_1 was dissolved in a mixture of deuteriochloroform and deuteriomethanol. The proton n.m.r. spectrum of this solution (Figure 1) clearly indicated the presence of aflatoxin B_1, aflatoxin D_1, and MW206. Certain groups of resonances, particularly the doublets due to the acetal protons, were well resolved using high field n.m.r. (360 MHz), and so observation of these resonances in a time course experiment would reveal the changes in concentration of the various constituents in the product mixture bearing the dihydrofurobenzofuran moiety.

The first method to be investigated allowed these changes to be monitored *in situ*, by observing the proton n.m.r. spectrum of a sample of aflatoxin B_1, dissolved in deuterated ammonium hydroxide.

In order to be able to distinguish between aflatoxin D_1 (2) and MW206 (3) in the product mixture, reference spectra were run (Table 1) for the pure compounds in deuterated ammonium hydroxide. For aflatoxin D_1, the most noticeable differences between this spectrum and that run in a mixture of deuteriochloroform and deuteriomethanol, were the rapid loss of the methylene reasonances by exchange, and the shift to lower frequency of the aromatic proton, presumably due to formation of the phenolate anion.[12] The aromatic proton also slowly exchanged, although the aromatic protons in the spectrum of MW206 exchanged much faster. Apart from the difference in

aromatic protons, the spectra of aflatoxin D_1 and MW206 were very similar.

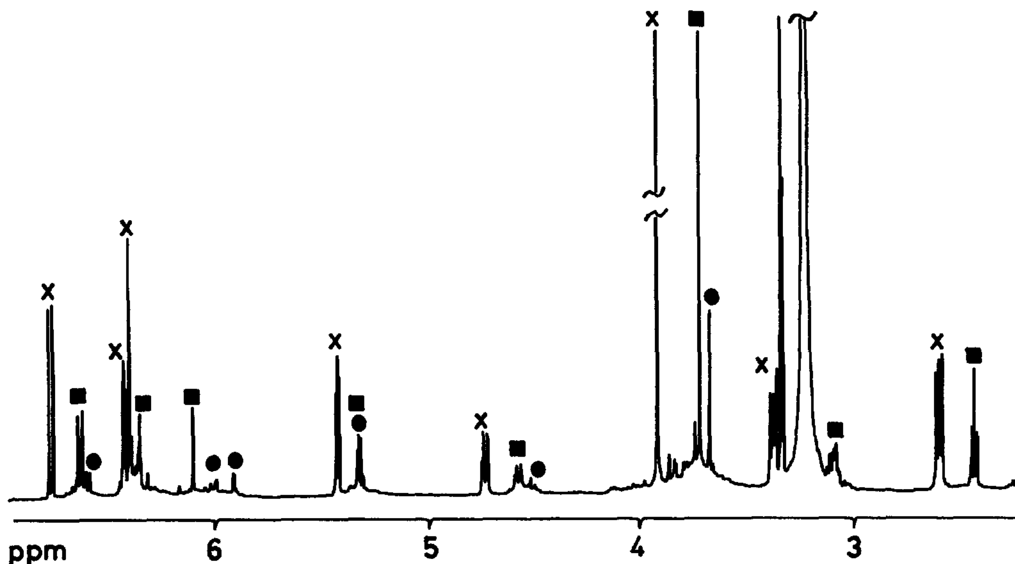


Figure 1 360 MHz ^{1}H N.m.r. spectrum of aflatoxin B_1 ammoniation product in $CDCl_3$-CD_3OD. Signals assignable to aflatoxin B_1 (X), aflatoxin D_1 (■) and MW206 (●) are indicated.

Therefore, a mixture of aflatoxin D_1 and MW206 (1.5:1 respectively) was analysed to verify their relative chemical shifts. It could be seen that the resonances for particular protons in aflatoxin D_1 were consistently 0.02-0.04 ppm to higher frequency than the corresponding ones in MW206, and that both sets of signals were present at chemical shift values identical to those obtained in the spectra of the pure compounds. With this information, the results of the n.m.r. studies of aflatoxin B_1, dissolved in ammonium hydroxide could be interpreted.

When aflatoxin B_1, was dissolved in deuterated ammonium hydroxide, the yellow-brown solution gave an initial proton n.m.r. spectrum in which the aromatic proton was at the same chemical shift as that in aflatoxin D_1, suggesting a phenol in a ring-opened form (6) of aflatoxin B_1. The spectrum taken after allowing the solution to stand at room temperature for 8 days (Figure 2a) indicated the presence of new resonances, which differed most from the original ones in the chemical shifts of the aromatic and methoxyl protons, which were both shifted 0.04 ppm to lower frequency. After 19 days (Figure 2b), the original resonances had disappeared completely, being replaced by the new ones seen on day 8. A minor methoxyl resonance was also visible, at the chemical shift consistent with MW206. One possible structure (7) for the major product after 19 days, is that derived by Michael addition of ammonia to the coumaric acid (6) (Scheme 2). There is a precedent for Michael addition to such systems, since the isolation of β-aminodihydrocoumaric acid (8) from the reaction of coumarin (9) with ammonium hydroxide has been reported.[13]

The sample was then heated at 50°C for 10 days, whereupon it turned

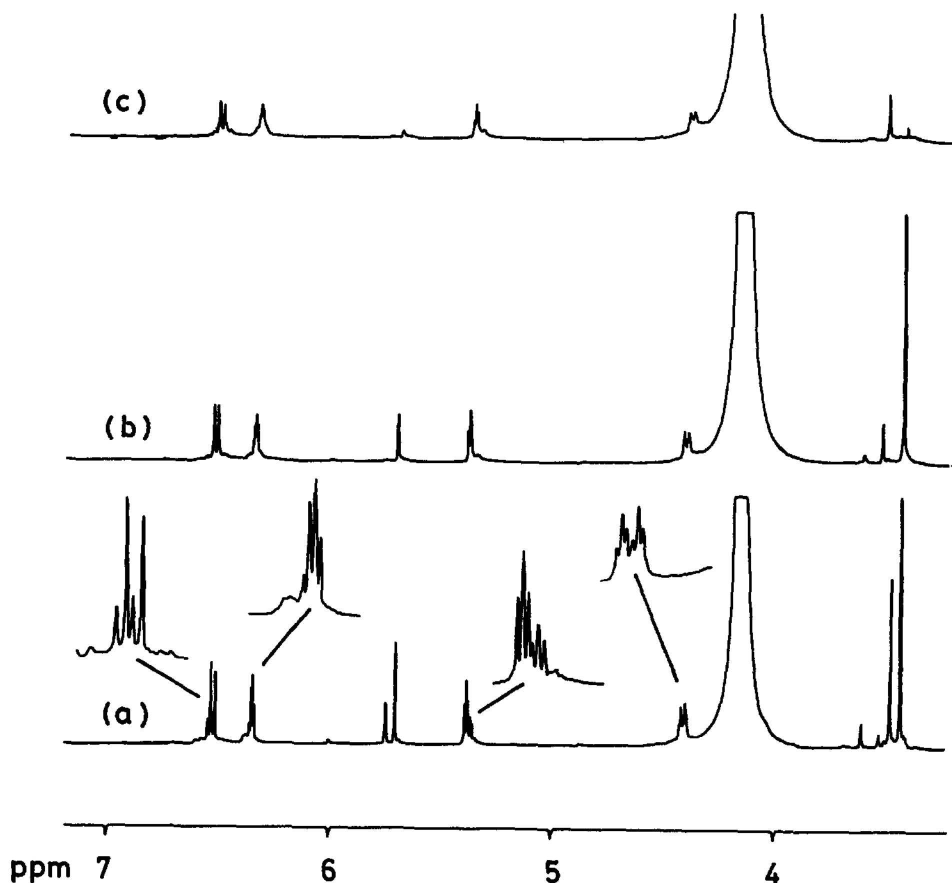


Figure 2 360 MHz ^{1}H n.m.r. spectrum of aflatoxin B_1 dissolved in ND_4OD after (a) 8 days, and (b) 19 days at room temperature, and (c) after a further 10 days at 50°C

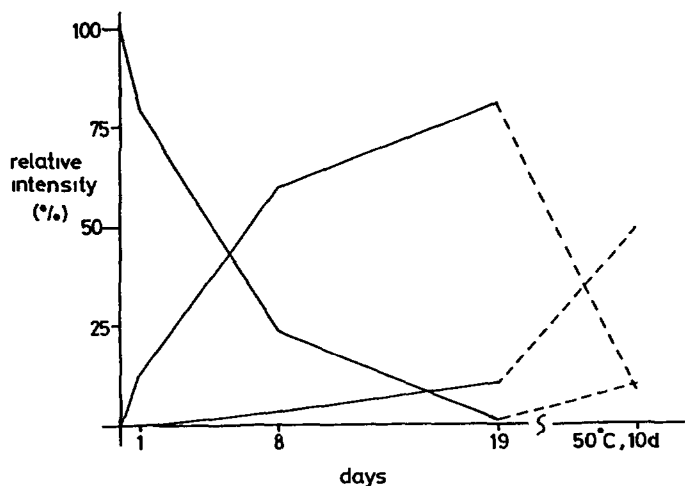
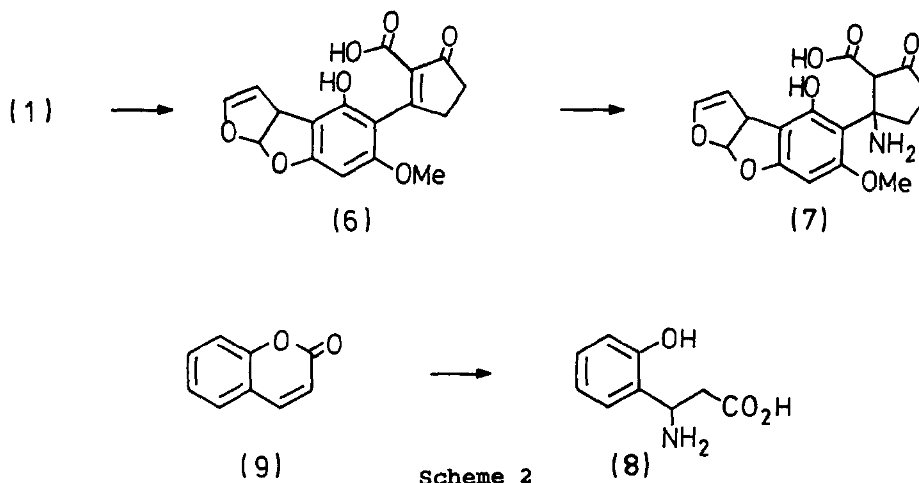


Figure 3 Relative intensities of methoxyl resonances due to products in the reaction of aflatoxin B_1 with ND_4OD at room temperature and after heating



Scheme 2

black. All the groups of resonances in the proton n.m.r. of the product (Figure 2c) showed a degree of line-broadening, and were poorly resolved. The main methoxyl resonance was at a chemical shift corresponding to that in MW206, and the other resonances which could be resolved were all more consistent with the presence of MW206, rather than aflatoxin D_1.

The graph (Figure 3) shows that the initially formed ring-opened product is slowly converted, at room temperature, to another form which still retains the dihydrofurobenzofuran system. Subsequent heating results in the appearance of a single major product, which corresponds closely to MW206. If this product is indeed MW206, then the anomalous production of this compound without the accompanying presence of aflatoxin D_1, must be due to the period of 19 days at room temperature before heating, and the formation of the second ring-opened compound (7).

Although this method seems to be the most desirable in that no work-up is required in order to observe the n.m.r. spectrum of the reaction mixture, it suffers the disadvantages that some signals are lost by exchange, and that the quality of the spectrum deteriorates on heating. The use of more standard solvents was therefore reinvestigated. Treatment of lyophilised ammoniation samples with a variety of solvents, resulted in pyridine being chosen as the most suitable n.m.r. solvent for subsequent analysis. To follow the ammoniation reaction over a period of time, a solution of aflatoxin B_1 in concentrated ammonium hydroxide was divided equally between a number of flasks, which were all sealed in an identical manner, and placed in an oven at 52°C. Samples were dissolved in deuteriopyridine and their proton n.m.r. spectra were obtained at 360 MHz (Figures 4 and 5). The spectrum of the aflatoxin B_1 sample used was also run (Figure 4a), and this indicated that an impurity of *ca* 10% aflatoxin B_2 (10) was present. Due to the resolution obtained at high field, this impurity did not interfere, but in fact as seen below gave a certain amount of valuable information on the products of ammoniation of aflatoxin B_2 at the same time.

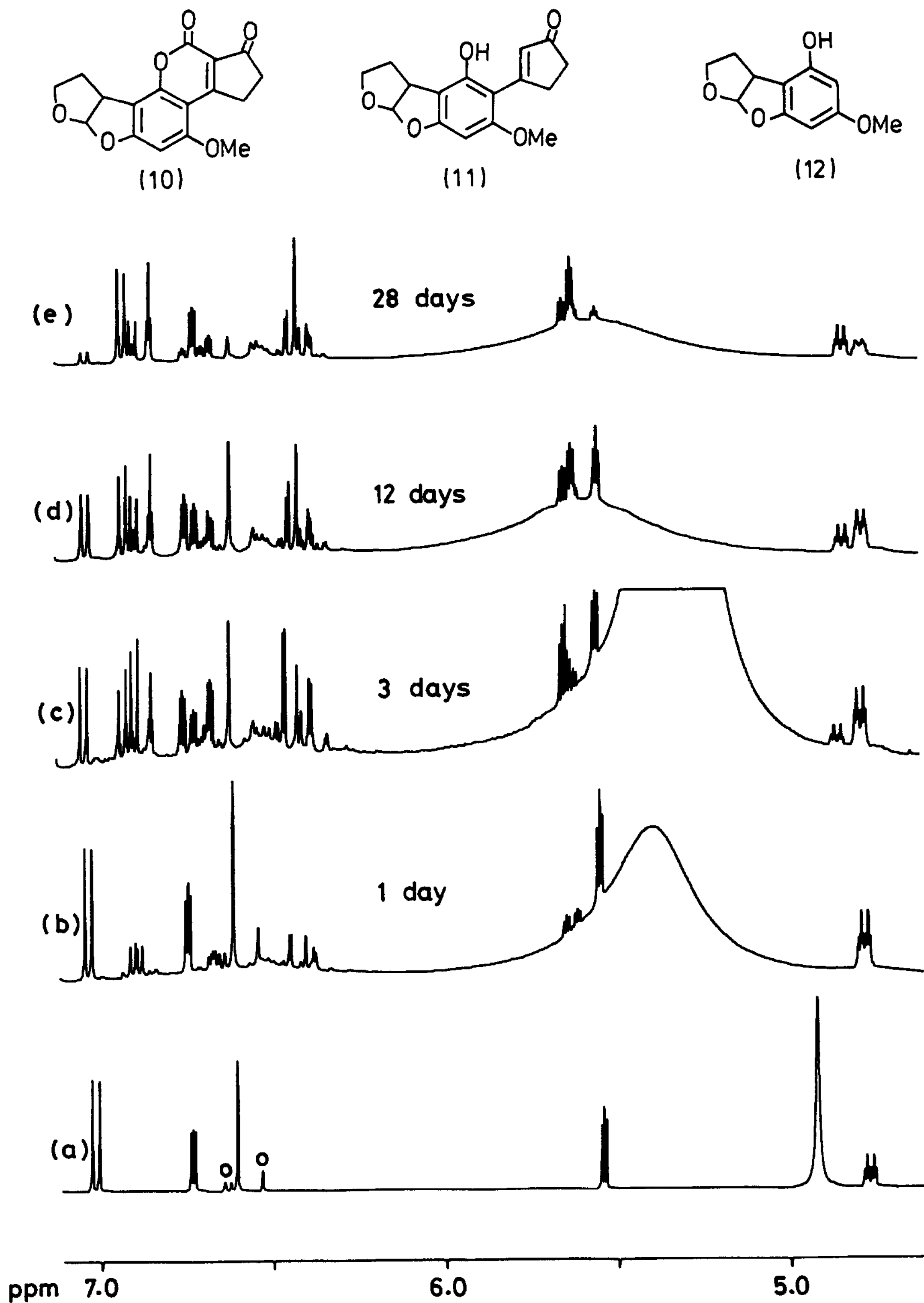


Figure 4 360 MHz ^{1}H n.m.r. spectra of the products of ammoniation of a mixture of aflatoxin B_1 (90%) and aflatoxin B_2 (10%) in d_5-pyridine

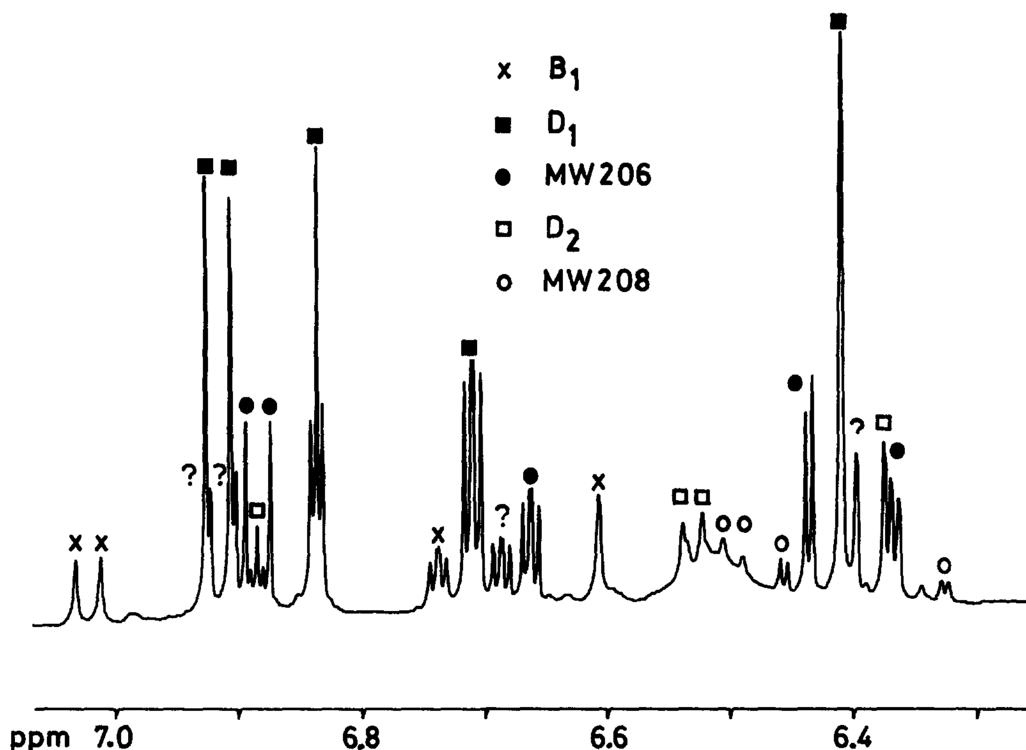


Figure 5 Expansion of the 360 MHz ^{1}H n.m.r. spectrum of the products of ammoniation of aflatoxin B_1 and aflatoxin B_2 after heating at 50°C for 28 days.

The area of the spectrum which gave the most information was that between 6.3 and 7.1 ppm, containing the acetal, aromatic, and certain olefinic signals. Figure 4 shows how this region changed with time. By observing the sets of resonances which increase or decrease in concert, between the spectra, it is possible to associate them with a single component. The signals corresponding to aflatoxin B_1 (*ie* the acetal doublet at 7.02 ppm, the olefinic doublet of doublets at 6.74 ppm, and the aromatic singlet at 6.61 ppm) can thus be seen to diminish with time. Similarly, those attributable to aflatoxin B_2 (ie the acetal doublet at 6.64 and the aromatic singlet at 6.54 ppm) also decrease.

One set of signals which gradually increases consists of an acetal doublet (J=7 Hz) at 6.92 ppm, an olefinic triplet (J=1.8 Hz) at 6.83 ppm, an olefinic doublet of doublets at 6.71 ppm, and an aromatic singlet at 6.41 ppm. This set clearly corresponds to aflatoxin D_1.

Another set of signals which increases at an initial rate faster than those due to aflatoxin D_1 can be ascribed to MW206. This consists of an acetal doublet at 6.88 ppm, an olefinic doublet of doublets at 6.66 ppm, and a pair of aromatic doublets (J=2 Hz) at 6.45 and 6.37 ppm. The lower frequency of the aromatic doublets experiences a broadening effect which the other doublet does not. This is presumably due to a long-range interaction with the methoxyl group.

Interestingly, there is another minor pair of doublets (at 6.47 and 6.32 ppm) which mirror this effect, and increase at a rate similar to those for MW206. It is reasonable to assume therefore, that this is the dihydro-analogue (11) of MW206 (MW208), derived from aflatoxin B_2. If so, there should also be a corresponding acetal doublet visible for this compound. There are indeed two possible doublets, which appear at 6.49 and 6.53 ppm, and have coupling constants of 5.6 Hz. The remaining doublet should therefore be due to the dihydro-analogue (12) of aflatoxin D_1, which may be called aflatoxin D_2. If aflatoxin D_2 is present, then an aromatic singlet and an olefinic triplet must also be present in the chemical shift range under investigation. There are two remaining unassigned singlets, at 6.40 and 6.36 ppm. The latter signal grows at the same rate as that of aromatic proton of aflatoxin D_1 and so is likely to be due to the aromatic proton of aflatoxin D_2. The olefinic triplet is likely to be the minor one which appears at 6.88 ppm (Figure 5).

A final minor set of resonances which is visible at day 1 and remains relatively constant throughout consists of an acetal doublet at 6.91 ppm, an olefinic doublet of doublets at 6.68 and the singlet at 6.40 ppm. These resonances indicate that this compound contains the dihydrofurobenzofuran moiety, and an aromatic ring similar to that of aflatoxin D_1. It is possible that these signals could be due to a steady state concentration of the ring opened coumaric acid (6) or the corresponding Michael addition product (7) discussed above.

Having assigned the spectra, the changes in relative concentration of the various components were estimated by observing the change in intensities of the signals corresponding to those components (Figure 6). This clearly showed a decrease in aflatoxin B_1 concentration, and an increase in aflatoxin D_1 and MW206. The trace for MW206 shows that it is not derived from aflatoxin D_1, and it also shows an interesting maximum at day 8. If this maximum is not merely due to experimental error, it may be due to one or both of the following:

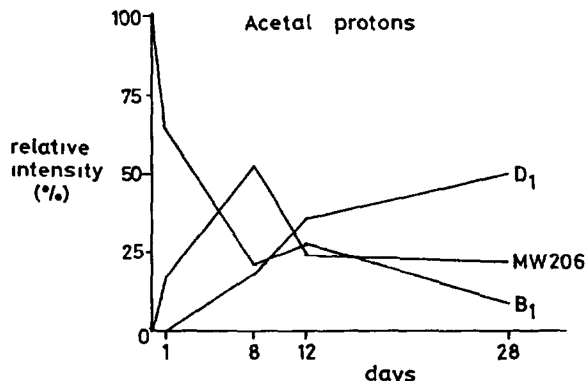
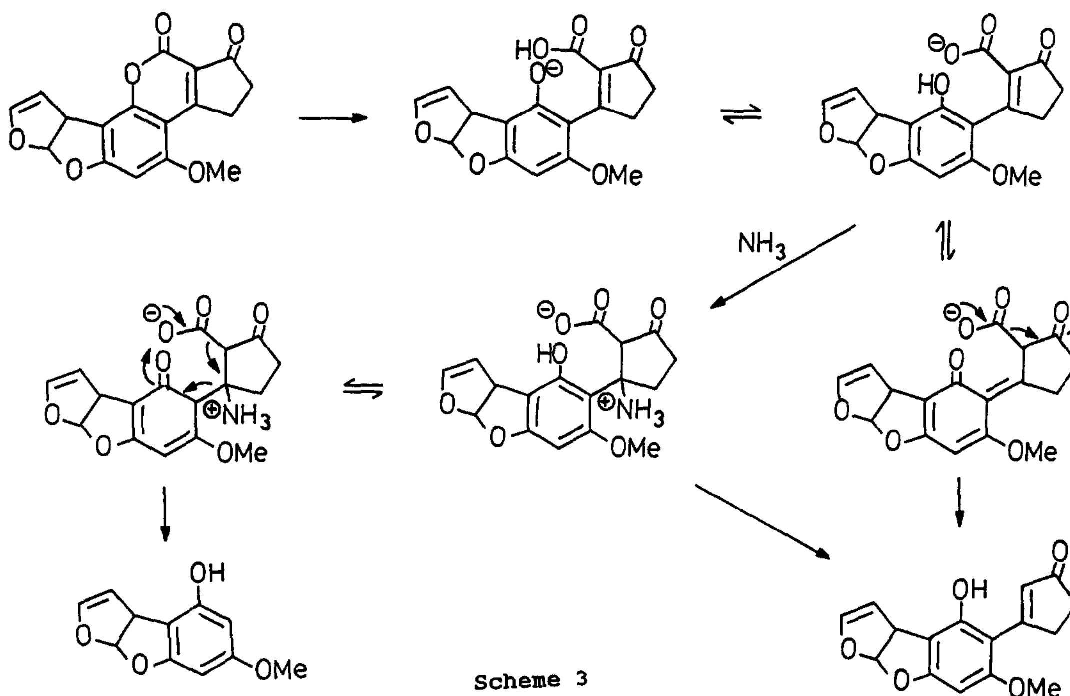


Figure 6 The variation in the relative intensities of the acetal proton resonances corresponding to aflatoxin B_1, aflatoxin D_1, and MW206 during the ammoniation of aflatoxin B_1



Scheme 3

(a) The rate of production of MW206 is faster than that of aflatoxin D_1, but it is then further degraded, whereas aflatoxin D_1 is not.

(b) The seal on the flasks may not have been perfect, so there may have been a gradual decrease in the concentration of ammonia due to leakage.

Aflatoxin D_1 can be considered as arising from a unimolecular decarboxylation of a ring-opened form of aflatoxin B_1 as shown in Scheme 3, but in order to write a mechanism for the formation of MW206, it is necessary to invoke a Michael addition type process. Therefore, the rate of production of MW206 will be faster at the start of the experiment, when the concentration of ammonium hydroxide is at its greatest.

Also, the fact that MW208 and aflatoxin D_2 production mirrors that of MW206 and aflatoxin D_1, confirms the assumption that only the coumarin system is involved in the degradation of pure aflatoxins by ammonium hydroxide.

The above experiments show that high field n.m.r. analysis of ammoniation mixtures can indeed be used to monitor the course of the reaction, and also to give structural information on the products as they appear. It is likely that with the use of decoupling experiments and more detailed analysis of the spectra, yet more information could be obtained. However, since this experiment requires a work-up step, a well-planned, standardised procedure should be adopted to minimise the chance of the production of artefacts. Further work on the effect of temperature on the production of aflatoxin D_1 and MW206 would be desirable, and in particular, the effect on product distribution of a time-delay before commencing heating would be interesting.

Experimental

Melting points were determined using a Reichert hot stage microscope and are uncorrected. Analyses were determined on a Carlo Erba 1106 elemental analyser. Infra-red spectra were obtained on a Perkin-Elmer 781 spectrophotometer. Ultra-violet spectra were determined on a Pye Unicam SP8-400 spectrophotometer. Proton n.m.r. spectra were obtained on Varian EM360, Bruker WP80SY, WP200SY, and WH360 instruments. All quoted chemical shifts are relative to tetramethylsilane, δ_H = 0.00 ppm. Mass spectra were obtained on Kratos MS 902 and MS 50 TC spectrometers. Thin layer chromatography was carried out on either analytical (5 x 20 cm) or preparative (20 x 20 cm) glass plates, coated with a 0.5 mm layer of silica gel (Fluka AG 60765 Kieselgel GF_{254}). Chromatograms were visualised under ultra-violet light (254 nm), by development in an iodine tank, or by spraying with a 1% solution of Fast Blue B in water.

Isolation of Aflatoxin B_1 (1) and Aflatoxin G_1

Aspergillus flavus 120920 spores were maintained under liquid paraffin on Czapek-Dox agar slopes. The slopes were used to inoculate three 500 ml Erlenmeyer flasks containing "low salts" medium[14] and grown for 3 d, on a rotary shaker at 26°C. This inoculum was then used to inoculate 2 l of "low salts" medium distributed between 14 x 500 ml Erlenmeyer flasks, and incubated for 7 d, at 26°C on a rotary shaker. The contents of the culture flasks were filtered, and the liquors were reserved. The mycelium was homogenised with acetone in a Waring blender, then filtered. The process was repeated twice on the residual mycelium, and the combined acetone extracts were concentrated *in vacuo*, leaving an orange, aqueous solution. This solution was extracted with chloroform (3 times), as was the previously reserved liquors layer, and the extracts were combined, then concentrated *in vacuo*. The residual solid was dissolved in a mixture of methanol and water (9:1), and washed with (40-60) petroleum ether (2 times). The polar layer was then concentrated *in vacuo*, to an aqueous solution, which was extracted with chloroform (3 times). The chloroform layer was dried ($MgSO_4$), and concentrated *in vacuo*, to give the crude extract (389 mg). The crude extract was purified by preparative tlc (71% chloroform - 12% acetone - 17% (40-60) petroleum ether), and the major blue fluorescent band gave aflatoxin B_1 (1) (125 mg; 62 mg/l). The major green fluorescent band gave aflatoxin G_1 (64) (70 mg; 35 mg/l). δ_H (80 MHz; $CDCl_3$) 3.42 (2H, t, J 6 Hz), 3.92 (3H, s), 4.40 (2H, t, J 6 Hz), 4.75 (1H, dt, J 7.2 Hz), 5.45 (1H, t, J 2 Hz), 6.40 (1H, s), 6.45 (1H, dd, J 3.2 Hz), 6.79 (1H, d, J 7 Hz) ppm.

Initial Ammoniation of Aflatoxin B_1 (1)

Concentrated ammonium hydroxide (s.g. 0.88; 10 ml) was added to aflatoxin B_1 (1) (20 mg, 0.06 mmol), and the mixture was heated at reflux overnight. The solution was then neutralised by the addition of 2 M HCl, then extracted with ethyl acetate. The organic layer was then dried ($MgSO_4$), and concentrated *in vacuo* to a solid. Analytical tlc of the product (2% methanol - 98% chloroform) revealed aflatoxin B_1 at R_f 0.33, and a non-fluorescent spot at R_f 0.26. The non-fluorescent material was separated by preparative tlc (2% methanol - 98% chloroform, then 10% methanol - 90% chloroform) to give aflatoxin D_1 (2) (6 mg, 0.02 mmol; 33%).

Treatment of Sterigmatocystin (4) with Ammonium Hydroxide

A mixture of sterigmatocystin (4) (52 mg, 0.16 mmol) and concentrated ammonium hydroxide solution (s.g. 0.88; 10 ml) was heated at reflux overnight. The solution was then neturalised by the addition of 2 M HCl, then extracted with ethyl acetate. The organic layer was dried ($MgSO_4$), then concentrated *in vacuo*, to an orange solid. The solid was purified by preparative tlc (5% methanol - 95% chloroform). The orange band gave unreacted sterigmatocystin (50 mg, 0.15 mmol; 96%).

Typical Reaction of Aflatoxin B_1 (1) with Ammonium Hydroxide Solution

To aflatoxin B_1 (76 mg, 0.14 mmol) in a round-bottomed flask was added

concentrated ammonium hydroxide solution (s.g. 0.88; 20 ml), and the flask was sealed and placed in an oven at 50°C for 21 d. The contents of the flask were then lyophilisised, and separated by preparative tlc (7% acetone - 93% methylene chloride). The lower non-fluorescent band gave aflatoxin D_1 (2) (40 mg, 0.14 mmol, 57%). The blue fluorescent band gave residual aflatoxin B_1 (1) (11.3 mg, 0.04 mmol, 15%), and the upper non-fluorescent band gave MW206 (5) (4.7 mg, 0.02 mmol, 9%).

Aflatoxin D_1 acetate (5)

(a) Aflatoxin B_1 (1) (26 mg, 0.08 mmol) was transferred to a round-bottomed flask, and concentrated ammonium hydroxide solution (s.g. 0.88; 10 ml) was added. The flask was stoppered and sealed, then placed in an oven at 57°C for 14 days. The contents were then lyophilised, and the product was subjected to preparative tlc (10% methanol - 90% methylene chloride), the non-fluorescent at R_f 0.47 giving aflatoxin D_1 (2) (10.2 mg, 0.04 mmol, 43%). A sample of this product was derivitised by heating for 1 h, at 110°C, with pyridine (1 ml) and acetic anhydride (1 ml). The solution was then poured onto ice and extracted with ethyl acetate. The organic layer was dried ($MgSO_4$) and concentrated *in vacuo* to a solid which was purified by preparative tlc (4% acetone - 96% chloroform), the blue fluorescent band giving aflatoxin D_1 acetate (5).

(b) Aflatoxin B_1 (1) (52 mg, 0.17 mmol) and concentrated ammonium hydroxide solution (s.g. 0.88; 20 ml) were placed in a flask, which was sealed and heated at 58°C for 21 d. The product was lyophilised, then dissolved in water (50 ml). The solution was acidified (2 M HCl), then extracted with ethyl acetate. After drying ($MgSO_4$), and concentration *in vacuo*, the organic layer yielded a solid, which was heated at 100°C for 75 min, with pyridine (2 ml) and acetic anhydride (1 ml). The solution was then poured onto a mixture of ice (20 mg) and 2 M hydrochloric acid (20 ml), then extracted with chloroform. The chloroform extract was washed with water, then dried ($MgSO_4$), and concentrated *in vacuo*, to a solid. This solid was then subjected to preparative tlc (10% acetone - 90% chloroform). The blue fluorescent band was removed, and further purified by repeated preparative tlc (5% acetone - 95% methylene chloride), giving aflatoxin D_1 acetate (5) (20 mg, 0.06 mmol, 36%), which gave fine needles from ethanol, m.p. 158-160°C (lit.[5] m.p. 155-157°C), (M^+: 328.09464. $C_{18}H_{16}O_6$ requires 328.09469); ν_{max} ($CHCl_3$) 1750, 1700, 1630, 1600 cm^{-1}; λ_{max} (MeOH) 227, 288, 316 nm (ϵ 12600, 4700, 6800).

Analysis of an aflatoxin B_1 (1) ammoniation mixture by proton n.m.r. spectroscopy

Aflatoxin B_1 (1) (50 mg) was added to concentrated ammonium hydroxide (20 ml) in a round-bottomed flask. The flask was stoppered and sealed, then placed in an oven at 41°C for 25 d. The product was lyophilised, then hot ethyl acetate (20 ml) was added. The mixture was filtered, and the extraction was repeated, then the combined filtrates were concentrated *in vacuo* to a solid (33 mg). This solid was dissolved in a mixture of $CDCl_3$ and CD_3OD, for ^1H n.m.r. spectroscopy (Figure 1).

Proton n.m.r. spectroscopy of aflatoxin B_1 (1), dissolved in deuterated ammonium hydroxide

Aflatoxin B_1 (1) (7 mg) was dissolved in d_4-ammonium hydroxide solution (33% in D_2O; 1 ml), and a sample (0.5 ml) was removed to an n.m.r. tube. This was analysed by ^1H n.m.r. after 1 day, 8 days (Figure 2a) and 19 days (Figure 2b) at room temperature. A sample (0.5 ml) was then placed in a round-bottomed flask, then stoppered and sealed. The flask was heated in an oven at 50°C for 10 d, then the product mixture was removed to an n.m.r. tube, and its proton n.m.r. spectrum was recorded (Figure 2c).

Time-course study of the ammoniation of aflatoxin B_1 (1), using ^1H n.m.r. spectroscopy

A sample of aflatoxin B_1 (1) [53 mg, containing aflatoxin B_2 (10) (*ca*

10%)] was dissolved in concentrated ammonium hydroxide (s.g. 0.88; 25 ml) and 5 ml aliquots were placed in five 25 ml round-bottomed flasks. The flasks were stoppered and sealed, then placed in an oven at 52°C. Flasks were removed after 1, 8, 12, 20, and 28 days, and lyophilised. All the samples, except for day 20, dissolved fully in d_5-pyridine, and were analysed by ^{1}H n.m.r. (Figures 4,5).

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