The Structure of Citrinin *in vivo*

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^1H- and ^{13}C-N.m.r. spectroscopy have been used to show that in aqueous solution at physiological pH the antibiotic citrinin exists not as the quinone methide (1) but as a diastereoisomeric mixture of hydrates. The equilibrium between the two diastereoisomers is slow, suggesting that citrinin, which is believed to interact with deoxyribonucleic acid (DNA), does not act as a simple Michael acceptor *in vivo*. Neither does citrinin intercalate into DNA.

The polyketide antibiotic citrinin is produced abundantly by *Penicillium citrinum* and a number of *Aspergillus* strains. It is routinely isolated by precipitation from the aqueous culture broth by acidification [1] and has been fully characterised in this form [2,3] as the quinone methide (1). Postulates about its mode of biosynthesis have included an enzymic dehydration step.[4]

(1)

Citrinin is reported to be carcinogenic;[5] it has been shown to inhibit transcription[6] and there is evidence that it interacts with DNA[5] although the molecular basis of the proposed interaction has not been extensively studied.

The quinone methide structure (1) suggests that citrinin might act as a Michael acceptor or intercalate into DNA. We now show that, in aqueous solution, citrinin exists as a mixture of diastereoisomeric hydrates which do not intercalate into DNA, and cannot act as simple Michael acceptors.

Methods and Results

During experiments to monitor citrinin production *Penicillium citrinum* was grown from spores on Czapek-Dox liquid medium. After the mycelium was firmly established (8 days) the medium was replaced by fresh medium of the same mineral composition but containing no carbon source and made up with 80% D_2O. (A certain amount of glucose, stuck to cell surfaces, did persist.) Citrinin production was monitored by ^1H n.m.r., removing small aliquots of medium at intervals over the next 5 days.

After 24 hours the n.m.r. spectrum showed peaks closely resembling the ^1H n.m.r. spectrum of citrinin in organic solvents, except that every peak was doubled and the peak due to 1-H was shifted more than 2 p.p.m. upfield (see Table 1). A simple doubling might have been due to interconversion of (1) with the alternative tautomer (2) which has been reported in the crystal,[7] but the chemical shift of 1-H is not consistent with this. The possibility of an equilibrium between free citrinin and a complex containing a divalent cation, or between two complexes was also considered, but the ^1H n.m.r. spectra of citrinin in phosphate buffer (D_2O) at pH 7.4 in the presence and in the absence of Mg^{2+} ions were essentially identical with one another and with the spectrum of citrinin in culture medium. The marked change of chemical shift of 1-H on going from organic to aqueous solution suggests that citrinin undergoes Michael addition of water to give a mixture of diastereoisomers (3).

Table 1. ^1H N.m.r. spectra of citrinin in organic and aqueous solutions

δ	$CDCl_3$	D_2O (major isomer)	D_2O (minor isomer)
1-H	8.24s	6.00s	5.94s
3-H	4.78q, *J* 6.8 Hz	4.10quint, *J* 6.5 Hz[a]	4.12q, *J* 7 Hz[a]
4-H	2.99q, *J* 7.3 Hz	2.75quint, *J* 6.5 Hz[a]	2.84q, *J* 7 Hz[a]
9-H_3	1.35d, *J* 6.8 Hz	1.25d, *J* 6.4 Hz	1.26d, *J* 6.9 Hz
10-H_3	1.23d, *J* 7.3 Hz	1.18d, *J* 6.8 Hz	1.08d, *J* 7.0 Hz
11-H_3	2.02s	2.01s	2.02s

[a] Overlapping signals: coupling constants given to 0.5 Hz.

Table 2. ^{13}C N.m.r. spectra of citrinin in organic and aqueous solutions

δ	$CDCl_3$	D_2O (major isomer)	D_2O (minor isomer)
C-1	161.95	87.26	86.55
C-3	80.91	70.97	73.30
C-4	33.72	36.04	34.50
C-4a	139.17	143.75	142.51
C-5	122.01	112.69	112.17
C-6	182.73	158.39	158.19
C-7	99.36	111.61	110.91
C-8	176.15	155.76	156.16
C-8a	106.44	100.73	101.08
C-9	17.56	18.41	21.13
C-10	8.72	10.43	8.83
C-11	17.83	18.94	19.27
C-12	173.48	176.78	176.78

(2) (3)

Further evidence for this was obtained from the ^{13}C n.m.r. spectrum of citrinin in aqueous buffer. The peak due to C-1 which can easily be identified in the off-resonance spectrum in which it appears as a doublet, is upfield shifted by about 70 p.p.m. (see Table 2). The peaks due to C-7 and C-5 show small upfield shifts. Some uncertainty arose because there was only one carboxyl signal and there was an extra peak at δ 112 (where a small impurity often appears). The ^{13}C n.m.r. spectrum of

citrinin enriched with ^{13}C in C-10, C-11, and C-12 was, therefore, run under the same conditions. Only five peaks were enhanced—those due to C-10 and C-11 and the single carboxyl peak. It was concluded that the peaks due to C-12 of the two diastereoisomers are coincident.

From the integrals in the ^{1}H n.m.r. spectrum it can be seen that the two stereoisomers (**3**) are present in a ratio of about 2:1. The 3-H, 4-H coupling constants are quite different, about 6.5 Hz in the major isomer and negligible in the minor isomer, but it is not possible from these data alone to make confident predictions about which of the two structures (**3**) represents the major and which the minor isomer.

On heating to 90 °C there was no change in the ^{1}H n.m.r. spectrum, even the Me signals at δ 2.0 showed no sign of coalescence. The two compounds could not, however, be separated by preparative chromatography although it is possible to obtain two poorly resolved spots on t.l.c. in chloroform–methanol–aqueous ammonia (70:25:5) on silica gel.

Citrinin was tested for intercalation into DNA by u.v. spectral shift determination in the presence of DNA.[8] No evidence of intercalation or of any other direct interaction was observed, as no bathochromic shift in the wavelength maximum of citrinin in the presence of DNA (which is indicative of intercalation) could be demonstrated.

Discussion and Conclusions

In aqueous solution at physiological pH citrinin exists as the hydrate of the structure in chloroform solution. In the extracellular medium, though not necessarily within the cell, both diastereoisomers exist, in a 2:1 ratio. These results suggest a means by which citrinin may pass through cell membranes. Citrinin hydrate is produced inside the cell, and, if the hydration is under enzymic control, may be stereochemically pure. At the cytosol-membrane interface, dehydration to give the lipid soluble citrinin (**1**) will occur and after transport through the membrane, rehydration will give the observed mixture of stereoisomers in the culture medium.

The two isomers are in very slow exchange as evidenced by the n.m.r. results. Although interconversion of the two isomers does not require the formation of the quinone methide, formation of the quinone methide would lead to interconversion of the isomers. Thus formation of the quinone methide in aqueous media is unfavourable suggesting that the biological activity of citrinin is not a result of a simple Michael addition in free solution, although formation of the quinone methide in an enzymic hydrophobic pocket followed by Michael addition is possible. We have also shown that citrinin does not intercalate into DNA since there is no bathochromic shift in the u.v. spectrum of citrinin in the presence of DNA. These results are in accord with the observation that 1-methyl-citrinin has negligible antibacterial activity[9] and with the suggestion that the effect of citrinin on DNA is secondary.[10]

Experimental

Penicillium citrinum was grown and citrinin extracted as previously described.[1] Citrinin labelled with ^{13}C in C-10, C-11, and C-12 was also prepared as described.[1]

^{1}H N.m.r. spectra were determined on a Bruker WH300 spectrometer and ^{13}C n.m.r. spectra on a Bruker AM 250 spectrometer operating at 300 and 63 MHz respectively. Spectra were determined in 60mM sodium phosphate buffer at pH 7.4 in D_2O. ^{1}H N.m.r. spectra are referenced to δ 2.02 for the 11-H_3 peak taking the value for the spectrum in $CDCl_3$ solution, which is in turn referenced to tetramethylsilane. ^{13}C N.m.r. spectra are referenced to $CDCl_3$, which is referenced to tetramethylsilane.

U.v. spectra were recorded using a Cecil CE 202 spectrophotometer and against a blank of 0.05M NaCl–0.008M Tris chloride buffer at pH 7.0. Five solutions in 0.05M NaCl–0.008M Tris chloride buffer at pH 7.0 containing the same concentration of citrinin and varying amounts of DNA (Calf Thymus, Sigma Type 1) were prepared. The DNA:citrinin ratios were 0, 1, 2, 5, and 15 respectively. The spectra over the range of the maximum absorbance (280—380 nm) were recorded superimposed upon each other.

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