Proton Nuclear Magnetic Resonance Evidence for Two Configurations of the Hemiacetals of Aflatoxin B_1 and Sterigmatocystin

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The products of the acid-catalyzed addition of water across the vinyl ether double bond in aflatoxin B_1 and sterigmatocystin were characterized by proton nuclear magnetic resonance spectroscopy. Using a series of homodecoupling experiments, we identified and determined both configurations of the hemiacetals semiquantitatively. In addition, coupling constants determined in this study were used to give the approximate spatial conformation of the protons bound to the hemiacetal ring system.

The aflatoxins and sterigmatocystins are fungal metabolites produced by certain strains of the molds *Aspergillus flavus*, *Aspergillus parasiticus*, and *Aspergillus spp.* (Cole and Cox, 1981). These substances are a considerable public health concern because of acute and chronic toxic responses and carcinogenic effects resulting from exposure to them and their widespread occurrence in animal and human foodstuffs. Aflatoxin B_1 is the most potent of the aflatoxins and has been the object of studies aimed at developing fluorescence methods for its determination (Takahashi, 1977; Hutchins and Hagler, 1983).

Aflatoxin B_1 contains a bisfuran ring system and has been shown to be readily converted to the much less toxic hemiacetal derivative by the acid-catalyzed addition of water across the vinyl ether double bond (Andrellos and Reid, 1964; Pohland et al., 1968), as shown in Figure 1. This derivative was found to have enhanced fluorescence and thus is a more desirable analyte than the native compound (Hutchins and Hagler, 1983). Formation of the hemiacetal has been suggested as a confirmatory test for aflatoxin B_1. If conditions for analysis of the hemiacetals are to be optimal, the products of the derivatization reaction must be fully characterized.

Nuclear magnetic resonance (NMR) is very useful in locating the position of new hydroxy groups (Case, 1973) and thus shows promise as a technique to identify the hemiacetal derivatives of mycotoxins. A number of previous proton NMR studies have characterized the NMR spectra of fungal metabolites. These studies have reported spectra on sterigmatocystin (Bullock et al., 1962), aflatoxin B_1 (van der Merwe and Fourie, 1963; Asao et al., 1965), and the hemiacetal of aflatoxin B_1 (Pohland et al., 1968). Bullock et al. (1962) used coupling constants and chemical shifts, which they determined, to make suggestions about the relative angles between some of the protons, but none of the other investigators have ventured suggestions about the configuration or molecular conformation of these molecules.

In this study, we describe proton NMR spectra for the mycotoxins aflatoxin B_1, sterigmatocystin, and their hemiacetals. The chemical shift data and coupling constants provide evidence for identification of two separate configurations of each of the hemiacetals. The study also gives suggestions about the spatial arrangement of the protons bound to the bisfuran moiety.

EXPERIMENTAL SECTION

Aflatoxin B_1 and sterigmatocystin were purchased from Sigma Chemical Co. (St. Louis, MO) and examined without further purification. The hemiacetals were prepared by acid-catalyzed addition of water to aflatoxin B_1 or sterigmatocystin. A 100-μg portion of the mycotoxin was reacted at 60 °C with 10 mL of hexane and 2.5 mL of trifluoroacetic acid. After 15 min, 950 μL of water–acetonitrile (9:1) was added, the solution vortexed, and the lower aqueous layer removed. This layer was dried by warming to 40 °C under a gentle stream of nitrogen before the material was redissolved in solvent for NMR analysis.

Proton NMR spectra were obtained on a Varian Associates XL-300 spectrometer equipped with a 7.0-T superconducting magnet and using the XL data system. All Fourier transform spectra were measured at an ambient temperature of 24 °C by using a spectral width of 4000 Hz and 30K data points. Approximately 100 μg of the mycotoxins or their hemiacetals was examined with deuteriochloroform as the solvent. Chemical shifts are reported downfield from tetramethylsilane against the residual chloroform signal at 7.25 ppm as reference. Selective homodecoupling experiments were performed using continuous-wave decoupling.

Table I. NMR Spectral Assignments for Aflatoxin B_1 and Sterigmatocystin

proton	chem shift, ppm	mult	coupling const, Hz
	Aflatoxin B_1		
1	6.47	dd	$J_{1,2}$ = 2.9
2	5.48	dd	$J_{1,3}$ = 2.2
3	4.76	ddd	$J_{2,3}$ = 2.4
4	6.81	d	$J_{3,4}$ = 7.1
5	6.42	s	
OCH_3	3.95	s	
7	3.40	m	
8	2.66	m	
	Sterigmatocystin		
1	6.50	dd	$J_{1,2}$ = 2.9
2	5.45	dd	$J_{1,3}$ = 2.0
3	4.82	ddd	$J_{2,3}$ = 2.4
4	6.83	d	$J_{3,4}$ = 7.2
5	6.44	s	$J_{7,8}$ = 8.4
OCH_3	3.99	s	$J_{7,9}$ = 1.0
7	6.76	dd	$J_{8,9}$ = 8.3
8	7.50	t	
9	6.83	dd	

RESULTS

Table I gives the proton NMR spectral assignments of aflatoxin B_1 and sterigmatocystin. These resonances could be assigned from chemical shifts, coupling constants, and the results of decoupling experiments. Measurements at high field (300 MHz) enabled the differentiation of certain coupling constants not possible at lower field.

The proton NMR spectrum of aflatoxin B_1 hemiacetal is given in Figure 2, and the results of homonuclear decoupling experiments used to make resonance assignments are given in Table II. The most obvious difference between this spectrum and the one for aflatoxin B_1 is the

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Table II. Aflatoxin B_1 Hemiacetal Homonuclear Decoupling Results[a]

decoupling freq, ppm	resonance freq, ppm								
	6.55	6.40	5.77	5.52	4.16	2.48	2.37	2.10	1.66
none	d (6.1)	d (6.0)	d (4.6)	d (4.7)	dd (6.1, 8.6)	d (14.0)	ddd (4.9, 9.0, 13.8)	ddd (4.0, 9.5, 13.0)	d (13.0)
6.55		d (5.8)	d (4.6)	d (4.4)	d (9.0)	d (13.2)	ddd (4.7, 9.0, 13.7)	ddd (4.5, 9.1, 13.3)	d (13.6)
5.77	d (6.0)	d (5.9)		d (4.2)	dd (6.2, 8.4)	d (13.4)	dd (9.4, 13.7)	ddd (4.5, 9.1, 13.1)	d (13.1)
5.52	d (6.2)	d (5.9)	d (4.6)		dd (6.1, 8.1)	d (13.7)	ddd (4.6, 9.1, 13.9)	dd (9.0, 13.4)	d (13.1)
4.16	s	d (5.9)	d (4.7)	d (4.7)		d (13.6)	dd (4.2, 13.6)	ddd (4.4, 8.9, 12.8)	d (13.1)
3.88	d (6.1)	d (1.9)[b]	d (4.7)	d (4.5)	dd (6.1, 8.8)	d (13.6)	ddd (4.6, 8.9, 13.7)	dd (4.4, 12.4)	d (13.1)
2.48	d (6.1)	d (6.0)	d (4.3)	d (4.4)	dd (6.3, 8.3)		dd (4.6, 8.0)	ddd (4.6, 9.3, 13.4)	d (13.1)
2.37	d (6.1)	d (6.0)	s	d (4.4)	dd (3.6,[b] 6.0)	s		ddd (4.2, 9.2, 13.0)	d (13.0)
2.10	d (6.1)	d (6.0)	d (4.7)	s	dd (6.2, 8.6)	d (13.7)	ddd (4.6, 9.2, 13.5)		s
1.66	d (6.1)	d (6.0)	d (4.6)	d (4.4)	dd (5.9, 9.1)	d (13.4)	ddd (4.7, 9.2, 13.8)	dd (4.2, 8.5)	

[a] Values given in parentheses are coupling constants (Hz). [b] Partially collapsed.

Table III. NMR Spectral Assignments for Hemiacetals

proton	chem shift, ppm	mult	coupling const, Hz
	Aflatoxin B_1 Hemiacetal		
1α[a]	5.77	d	$J_{1\alpha,2b\alpha} = 4.6$
1β	5.52	d	$J_{1\beta,2b\beta} = 4.7$
$2a\alpha$	2.48	d	$J_{2a\alpha,2b\alpha} = 13.9$
$2a\beta$	1.66	d	$J_{2a\beta,2b\beta} = 13.0$
$2b\alpha$	2.37	ddd	$J_{2b\alpha,3\alpha} = 8.8$
$2b\beta$	2.10	ddd	$J_{2b\beta,3\beta} = 9.5$
3α	4.16	dd	$J_{3\alpha,4\alpha} = 6.1$
3β	3.90	dd	$J_{3\beta,4\beta} = 6.0$
4α	6.55	d	
4β	6.40	d	
5α	6.35	s	
5β	6.24	s	
OCH_3	3.95	s	
7	3.41	m	
8	2.67	m	
	Sterigmatocystin Hemiacetal		
1α	5.78	d	$J_{1\alpha,2b\alpha} = 4.8$
1β	5.60	d	$J_{1\beta,2b\beta} = 5.0$
$2a\alpha$	2.53	dd	$J_{2a\alpha,2b\alpha} = 13.7$
$2a\beta$	1.81	d	$J_{2a\beta,2b\beta} = 13.2$
$2b\alpha$	2.42	ddd	$J_{2a\alpha,3\alpha} = 1.2$
$2b\beta$	2.22	ddd	$J_{2b\alpha,3\alpha} = 9.0$
3α	4.22	ddd	$J_{2b\beta,3\beta} = 9.2$
3β	3.95	dd	$J_{3\alpha,4\alpha} = 6.3$
4α	6.56	d	$J_{3\beta,4\beta} = 6.1$
4β	6.43	d	$J_{7,8} = 8.3$
5α	6.38	s	$J_{7,9} = 1.0$
5β	6.05	s	$J_{8,9} = 8.3$
OCH_3	3.98	s	
7	6.70	m	
8	7.50	m	
9	6.80	m	

[a] α and β indicate overall isomer configurations.

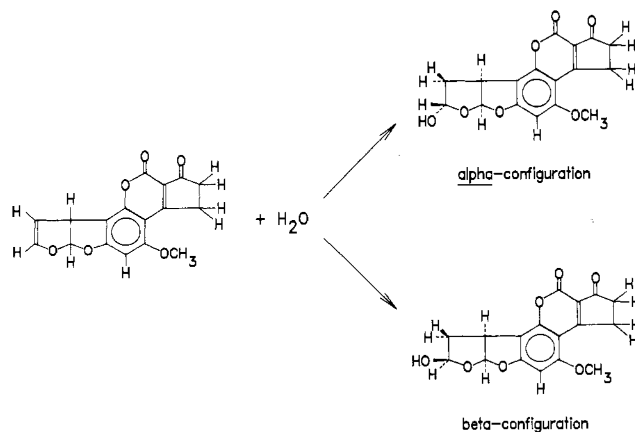

Figure 1. Acid-catalyzed addition of water across the vinyl ether double bond yielding α- and β-hemiacetal configurations.

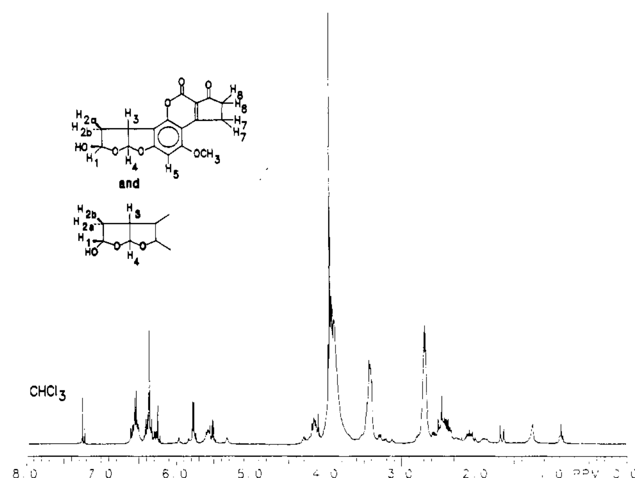

Figure 2. Proton nuclear magnetic resonance spectrum and structure of aflatoxin B_1 hemiacetals.

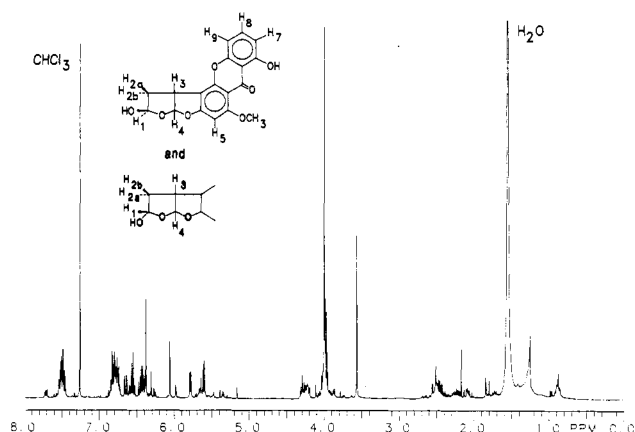

Figure 3. Proton nuclear magnetic resonance spectrum and structure of sterigmatocystin hemiacetals.

appearance of a greater number of resonances. Assignment of these resonances by inspection is a formidable task but can be accomplished by using homonuclear decoupling and by comparison with the underivatized mycotoxin. This proton spectrum shows two separate sets of resonances with similar coupling constants, but with different chemical shifts, suggesting the presence of more than one configuration of the hemiacetal. Table III summarizes assignments for both configurations of aflatoxin B_1 hemiacetal.

Results of proton NMR spectroscopy for sterigmatocystin hemiacetal (given in Figure 3) are similar to those for aflatoxin B_1 hemiacetal, and the assignment of resonances can be carried out in the same manner. These assignments also indicated two different configurations of the hemiacetal, as shown by two sets of distinct uncoupled

resonances. One difference between this spectrum and that of aflatoxin B_1 hemiacetal was the observation of a small (1-Hz) coupling between one set of H_{2a} and H_3 in the sterigmatocystin hemiacetal spectrum. In addition, the two different configurations resulted in different chemical shifts for protons H_7–H_9. The complexity of this part of the spectrum prevented complete spectral assignment, but the chemical shift effects are apparent.

DISCUSSION

The results given in Figures 2 and 3 suggest that hemiacetal formation did take place in this reaction and that more than one configuration was synthesized. The disappearance of allylic coupling between H_1 and H_3 indicates that the reaction involved the saturation of the double bond in the bisfuran ring system and that the conversion to the hemiacetal was complete. The shift in the H_2 resonances to the methylene region of the chemical shift range indicates that the hydroxy group must have added to position 1 and not to position 2.

The mycotoxins examined in this study are not planar molecules, so the addition of water across the double bond can result in two stereochemically distinct forms of the hemiacetal—an α configuration with the hydroxy group below the ring and a β orientation with the hydroxy group above the ring. The appearance of two sets of resonances in the spectrum of the hemiacetals indicates that both products were synthesized.

Figure 2 shows that the two forms of aflatoxin B_1 hemiacetal, α and β, were not equally produced, but occurred at about a ratio of ~70:30, respectively. Figure 3 shows that, in this case, the two configurations of α- and β-sterigmatocystin hemiacetal occurred at a ratio closer to 50:50. The ratio of α to β varies significantly with reaction and storage conditions. We have observed the disappearance of the α form of sterigmatocystin hemiacetal after a sample was stored for 6 months, but we have not yet identified the factors that control this conversion.

Many of the effects on protons of nearby hydroxyl groups in hexanose and furanose rings have been discussed by Casy (1971). Of particular interest to this study is the deshielding influence of axial hydroxy groups on axial protons in these rings. A similar condition exists in the α configuration of the hemiacetals of aflatoxin B_1 and sterigmatocystin. As shown in Figure 4, the hydroxy group is on the same side of the molecule as protons H_3 and H_4, and these protons should be deshielded relative to the β configuration in which this effect is not possible. H_3 and H_4 both occur at higher frequencies in one of the configurations determined in this study, and this allows the assignments given in Table III.

Karplus (1959, 1963) pointed out that the coupling between protons on vicinal carbon atoms in rigid systems is a function of the dihedral angle between the planes containing the bonds. As indicated in Figures 2 and 3 and Table III, there was no measurable coupling between H_1 and H_{2a}, which suggests that the dihedral angle between the bonds containing these protons is between 80° and 100°. For both the α and β configurations, this would result in an angle between H_1 and H_{2b} of 20–40°, and the observed coupling constant of 4.8 Hz is in general agreement with this result. In the β configuration of both hemiacetals the coupling constant between H_{2a} and H_3 was also immeasurable, so this angle must also be between 80 and 100°. This causes the angle between the bonds containing H_{2b} and H_3 to be between 20 and 40°. The coupling constant between these nuclei is ~9 Hz. The large difference between this value and $J_{1,2b}$ suggests that the angle between H_{2b} and H_3 is close to 20° and the angle between H_1 and H_{2b} approaches 40° in the β configuration.

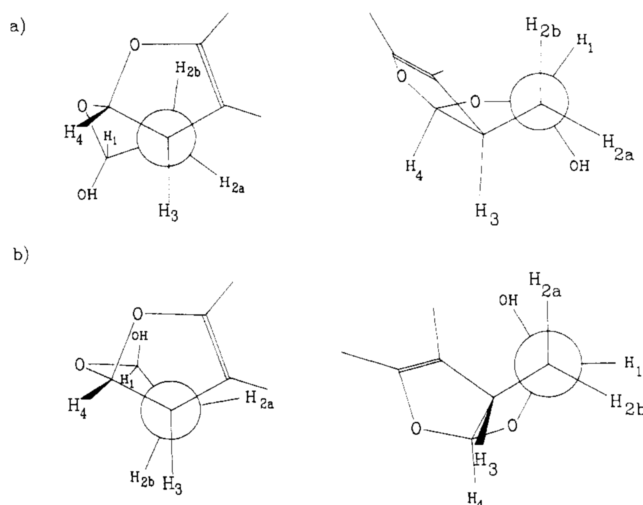

Figure 4. Newman projections of the (a) α-configuration and (b) β-configuration of the hemiacetal derivatives of aflatoxin B_1 and sterigmatocystin.

Results of sterigmatocystin hemiacetal given in Table III show a coupling constant of 1.2 Hz for $J_{2a,3}$ in the α configuration. This coupling could not be measured in the aflatoxin B_1 hemiacetal because of insufficient resolution but could be detected as a broadening of those resonances involved. The results for both molecules is consistent with a small coupling between H_{2a} and H_3 in the α configuration. Molecular models make clear the reason that this coupling is present in the α configuration. In the α configuration, it is not possible to obtain an angle between H_{2a} and H_3 of 80–100°. The maximum angle possible is ~50–60°, which is consistent with the determination of a small coupling between these protons. The angle between H_{2b} and H_3, therefore, must be about 170–180°. This result agrees with the measured coupling constant of about 8.9 Hz. The approximate structures of the α and β configurations of these two mycotoxins, as described by proton NMR coupling constants, are shown in Figure 4. These angles are only approximate and should not be considered as absolute. In spite of the qualitative nature of the analysis, the general molecular conformation is evident.

Some studies of the proton NMR spectra of mycotoxins have already been performed. Bullock et al. (1962) determined the proton NMR spectrum of sterigmatocystin in $CDCl_3$. The chemical shifts and coupling constants they reported show excellent agreement with the results given here. In addition, the value of $J_{3,4}$ was determined to be 6.9 Hz, which suggested that the molecule assumes a nonplanar-buckled conformation. The illustrations shown in Figure 4 suggest that buckled conformations are also found in the hemiacetal derivatives. Two groups have previously reported the spectrum of aflatoxin B_1 in $CDCl_3$ (van der Merwe and Fourie, 1963; Asao et al., 1965). Their results agree well with the values reported here, except for the determination of certain coupling constants. We used a 300-MHz frequency (instead of their 60 MHz) and were able to resolve $J_{1,2}$, $J_{1,3}$, and $J_{2,3}$ (2.9, 2.1, and 2.4 Hz, respectively). The proton NMR spectrum of aflatoxin B_1 hemiacetal was determined in pyridine-d_5 by Pohland et al. (1968). That study determined only a single configuration and made no attempt to assign this to the α or β configuration.

The results given in this study are in good agreement with previous studies of the proton NMR spectra of the mycotoxins aflatoxin B_1 and sterigmatocystin. We have

been able to show that, in the acid-catalyzed addition of water to these mycotoxins, both hemiacetal configurations and their approximate conformations can be determined by proton NMR spectroscopy. Since the proportions of these configurations vary with reaction conditions, any differences in fluorescence properties between the configurations should be determined before quantitative conclusions can be drawn from fluorescence measurements on the hemiacetal derivatives.

Registry No. Aflatoxin B_1, 1162-65-8; sterigmatocystin, 10048-13-2; aflatoxin B_1 hemiacetal (isomer 1), 17878-54-5; aflatoxin B_1 hemiacetal (isomer 2), 109278-37-7; sterigmatocystin hemiacetal (isomer 1), 109278-36-6; sterigmatocystin hemiacetal (isomer 2), 63324-97-0.

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Kinetics of Interaction of Aflatoxin M_1 in Aqueous Solutions Irradiated with Ultraviolet Energy

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Ultraviolet (UV) irradiation quenches fluorescence of aflatoxin M_1 (AFM_1) in aqueous media. Loss of the toxin and development of reaction products, in aqueous solutions, were monitored kinetically during the treatment with 365-nm, low-energy UV irradiation. Loss of AFM_1 followed a pattern similar to that of first-order reactions and was accompanied by production of a reaction product (AFM_x) that was more polar and more fluorescent than the parent compound (AFM_1). Reaction of AFM_1 was not affected by changes in pH of the reaction medium in the range pH 3–7. Stability of the reaction product (AFM_x), however, was affected by such changes in pH. Conversion of AFM_1 to AFM_x was only slightly affected by temperature in the range 0–60 °C (Q_{10} = 1.13). Further conversion of AFM_x by UV energy was influenced, to a greater extent, by the temperature of the reaction mixture during irradiation (Q_{10} = 1.35).

Aflatoxin M_1 (AFM_1) is a toxic and carcinogenic compound (Purchase and Vorster, 1968) that occasionally contaminates our food supply. There are two possible routes for contamination of food by this toxin: First, molds may produce AFM_1 while they are growing on food. Strains of *Aspergillus flavus* and *Aspergillus parasiticus* produced several aflatoxins including AFM_1 when they were grown on rice, groundnuts, maize meal, and cotton seeds (Purchase et al., 1968). AFM_1 has also been found, along with aflatoxin B_1 (AFB_1), as a natural contaminant in samples of stored corn (Shotwell et al., 1976). *A. parasiticus* NRRL 2999 was grown on bread and produced a significant amount of AFM_1 on that substrate (Reiss, 1981). Second, animals can be fed a ration contaminated with AFB_1, and then milk from such animals contains AFM_1 (Allcroft and Carnaghan, 1963; de Iongh et al., 1964).

There is concern about the potential hazard to the public health that is associated with presence of AFM_1 in milk. In the United States, milk containing AFM_1 at a level higher than 0.5 ppb should be removed from interstate commerce, an action that can inflict great economic losses on the dairy industry. In earlier publications (Yousef and Marth, 1985b, 1986) we reported the possibility of eliminating AFM_1 by treating milk with ultraviolet irradiation. To ensure the safety of UV-treated milk, however, AFM_1 reaction product(s) should be monitored during the course of treatment, indentified, and tested for possible toxicity. Since milk is a complex medium, monitoring AFM_1 reaction products can be a difficult task. In this study, an AFM_1 standard was simply dissolved in water, before it was irradiated. Reduction in the level of toxin and production of a fluorescent reaction product were studied kinetically during the course of treatment with UV energy.

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MATERIALS AND METHODS

Aflatoxin M_1 Standard. AFM_1 (Sigma, St. Louis, MO) was dissolved in chloroform (ChromAR, Mallinckrodt, Paris, KY) to give a primary standard solution of 1 μg/mL. To prepare an aqueous solution of the toxin, a measured volume of the primary standard solution was dispensed into a 20-mL beaker, chloroform was evaporated under a stream of N_2, and the toxin was redissolved in ca. 7 mL of deionized, glass-distilled water, with use of a magnetic stirrer. The aqueous solution of the toxin was quantitatively transferred to a 10-mL volumetric flask; volume was completed with water. To prepare a methanolic solution

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