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Nuclear Magnetic Resonance Identification of Versiconal Hemiacetal Acetate as an Intermediate in Aflatoxin Biosynthesis

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An intermediate in aflatoxin biosynthesis, previously identified as "versiconal acetate", was synthesized from labeled acetate using dichlorvos-treated cultures of *Aspergillus parasticus*. ^{13}C and ^{1}H FT NMR studies led to assignment of an alternate hemiacetal structure. Its biosynthetic relationship to aflatoxin is apparent from the labeling pattern found in this compound derived solely from acetate.

The aflatoxin-producing cultures of *Aspergillus parasiticus* when treated with the insecticide dichlorvos (dimethyl 2,2-dichlorovinyl phosphate) produce much less than normal amounts of aflatoxins (Schroeder et al., 1974; Yao and Hsieh, 1974), and instead produce primarily an orange pigment. It has been suggested that this pigment is an intermediate in aflatoxin biosynthesis (Yao and Hsieh, 1974; Singh and Hsieh, 1977); it has been tentatively identified as versiconal acetate (Schroeder et al., 1974). Our preliminary experiments (Yao and Hsieh, 1974) as well as those of Schroeder et al. (1974) could not unequivocally establish the structure of this compound.

We prepared this pigment from [1-^{13}C]-, [2-^{13}C]-, [1,2-^{13}C]-labeled and unlabeled sodium acetate. These samples were subjected to ^{13}C and ^{1}H pulsed Fourier transform NMR analysis in order to firmly assign a structure to this aflatoxin biosynthetic intermediate and to demonstrate its biogenetic relationship to aflatoxins. Our data indicate that the pigment is versiconal hemiacetal acetate (VHA) and is shown as structure **6** (Figure 1).

EXPERIMENTAL SECTION

Materials. [1-^{13}C] and [2-^{13}C]-labeled sodium acetate enriched in ^{13}C by 50–60 and 62%, respectively, were obtained from the International Chemical and Nuclear Corp., Chemical and Radioisotope Division, Irvine, Calif. The [1,2-^{13}C]-labeled sodium acetate 90% enriched was obtained from Merck and Co., Inc., Rahway, N.J. Dichlorvos was a gift from Shell Chemical Co. The

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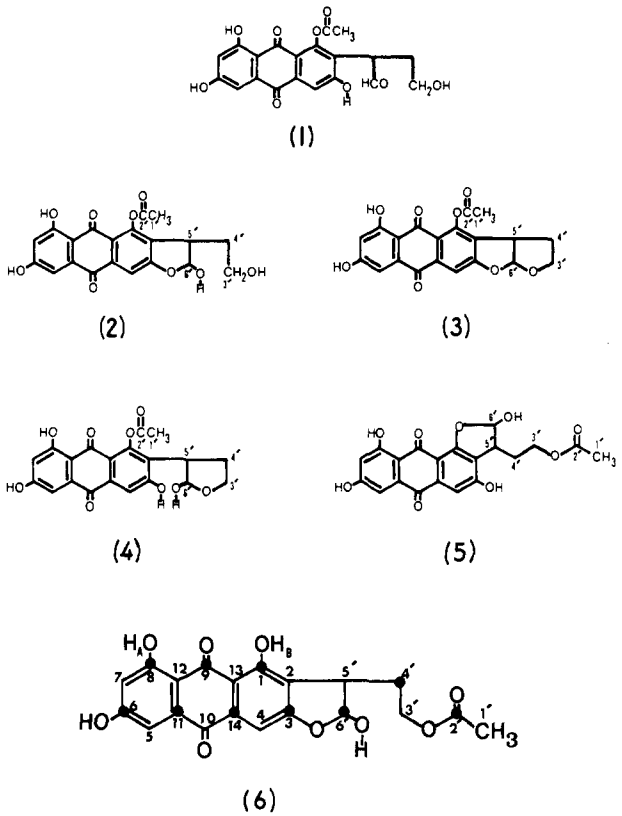

Figure 1. Possible structures for the orange pigment produced by dichlorvos-treated *A. parasiticus*: (1) versiconal acetate, the structure originally proposed by Schroeder et al. (1974); (2–5) other posssible structures for the compound; (6) versiconal hemiacetal acetate, the structure best indicated by our data; (●) carbons from C-1 of acetate, all others are from the methyl carbon of acetate. All protons are numbered the same as the carbons to which they are bonded.

acetone-d_6 was Lot A-1, obtained from Merck, Sharp and Dohme, Canada Limited, Montreal, Canada, and Diaprep 17, 595-1, a Division of Aldrich Chemical Co., Inc., Milwaukee, Wis. The chloroform-d_1 was Gold Label from Aldrich Chemical Co., Inc., Milwaukee, Wis. The methanol-d_4 was Lot D-323 from Stohler Isotope Chemicals, Rutherford, N.J.

Unlabeled versiconal hemiacetal acetate was obtained from *Aspergillus parasiticus* ATCC 15517 cultures grown in 100 mL of minimal mineral medium (Adye and Mateles, 1964) contained in 500-mL indented flasks. The cultures, at 30 °C, were shaken at 100 rpm for the first day and 200 rpm thereafter. Dichlorvos (10 ppm) was added to 48-h-old cultures and incubation continued for another 36 h. The orange pigment was extracted from cells and broth with acetone and chloroform, respectively.

^{13}C-labeled pigment from each of the above mentioned labeled precursors was obtained from cultures grown in a similar manner except that before addition of dichlorvos the 48-h-old mycelia from each flask were filtered and transferred to 500-mL indented flasks containing 50 mL of resting cell medium (Hsieh and Mateles, 1971) with the glucose level reduced to 1.62 g/L. Ten parts per million of dichlorvos was then added to each culture. Each of the ^{13}C-labeled precursors was added at 6-h intervals so as to result in a 4–6 mM solution per addition for a total of three additions. The pigment was extracted 6–10 h after the last addition.

Each of the crude pigment extracts was first partially purified by streaking it heavily (~10 mg/plate) on 0.25-mm precoated silica gel 60 TLC plates (EM Laboratories, Elmsford, N.Y.). These plates were repeatedly developed in chloroform ($R_f \simeq 0.0$) until no further bands eluted from the origin. The origin, containing the pigment, was scraped off and extracted with acetone. A final purification was accomplished by streaking the partially pure pigment again on silica gel 60 TLC plates which were developed in benzene–ethyl acetate–isopropanol–water (25:10:2:1, v/v/v, R_f 0.55). The identity of the purified compound was confirmed by mass spectral analysis.

Table I. ^{13}C NMR Spectral Data and Assignments of Versiconal Hemiacetal Acetate

δ, ppm[a]	Multiplicity[b]	Acetate carbon source[c]	Carbon assignment	Coupling constants J_{C-C}	Coupling constants J_{C-H}
20.7	q	2	1′	59	
29.4	t	1	4′		
44.6	d	2	5′		
62.8	t	2	3′		145
104.0	d	2	(4, 5, 7)	64	168
108.9	d	2	(4, 5, 7)	70	
109.9	s	2	(12, 13)		
110.5	d	2	(4, 5, 7)	64	163
111.5	s	2	(12, 13)		
116.2	d	1	6′		
121.6	s	2	2		
136.2	s	1	(11, 14)		
136.6	s	1	(11, 14)		
161.1	s	1	(1, 3, 6, 8)	64	
165.9	s	1	(1, 3, 6, 8)		
166.7	s	1	(1, 3, 6, 8)	70	
167.4	s	1	(1, 3, 6, 8)		
170.8	s	1	2′	59	
182.1	s	2	10		
190.5	s	1	9		

[a] In acetone-d_6. [b] Multiplicity determined from off-resonance proton-decoupled spectra: s, singlet; d, doublet; t, triplet; q, quartet. [c] 1, carbonyl; 2, methyl carbon.

Nuclear Magnetic Resonance Measurements. A JEOL PFT-100 Fourier transform NMR operating at 25 MHz (^{13}C spectra) and 100 MHz (^{1}H spectra) was used. Unless indicated otherwise, all spectra were obtained at an ambient probe temperature of ~28 °C. All chemical shifts are reported as δ (ppm) downfield from Me_4Si and in acetone-d_6 for the solvent. Most spectra were obtained from saturated solutions (~0.02 M). Instrumental parameters: 16 K data points, 6.25 kHz BDW, 6.25 kHz filter, 1–3 s repetition, 35–50° pulse angle, 10 000–30 000 pulses.

RESULTS AND DISCUSSION

Assignments of the ^{13}C and ^{1}H resonances for VHA were made from the spectra of [1-^{13}C], [2-^{13}C], [1,2-^{13}C], and unlabeled acetate-derived pigment. The chemical shifts (δ), their multiplicity, carbon sources, assignments, and coupling constants are shown in Tables I and II.

As can be seen from these data in Tables I and II, no aldehyde carbon or proton is apparent in the NMR spectra in acetone-d_6. For this reason, we have excluded the structure **1** proposed by Schroeder et al. (1974) for the orange pigment. Structures **2–6** might all give rise to similar carbon-13 and proton NMR spectra.

Structure **2** seems unlikely since it would require the H-3′-primary alcohol hydrogens to appear at δ 4.32. This type of primary proton should appear at ~δ 3.4 (Silverstein et al., 1974, appendix b). Structure **3** was eliminated because of a temperature dependence of the aliphatic proton (δ 2, 2.64, 6.47) and carbon signals (δ 29.4 44.6, 116.2) and the aromatic carbon signal to which the ali-

Table II. ^{1}H NMR Spectral Data and Assignments of Versiconal Hemiacetal Acetate and Emodin[e]

Compound	δ, ppm[a]	Multiplicity[f]	Integration	Assignment[b]
Versiconal hemiacetal acetate	12.24, 12.56[c]	s	~1	H_A, H_B
	7.32	d (J = 2.4 Hz)	1	H-5
	7.26	s	1	H-4
	6.72	d (J = 2.4 Hz)	1	H-7
	6.47	d	1	H-6′
	4.32	t (J = 6.6 Hz)	2	H-3′
	2.64	complex	1	H-5′
	~2	complex	*d*	H-4′
	~2.1	s	*d*	H-1′
Emodin[e]	12.16, 12.03	s	1.1 to ~2	H_A, H_B
	7.12	s	1	H-2
	7.52	s	1	H-4
	7.23	d	1	H-5
	6.67	d	1	H-7

[a] In acetone-d_6. [b] Structure 6. [c] At −50 °C. [d] Sum of H-4′ and H-1′ is ~5. [e] 1,6,8-Trihydroxy-3-methylanthraquinone. [f] s, singlet; d, doublet; t, triplet.

phatic side chain is attached (δ 121.6). The temperature dependence of these signals suggests the presence of two or more interchanging structures, which is inconsistent with a cyclic acetal group as in structure **3**.

In *o*-methylaversin, which has the same bisfuran ring system as in structure **3** (Bullock et al., 1963), the H-3′ protons and corresponding protons in a variety of other structurally similar compounds appear at δ 3.60–3.80 (Rodricks, 1969). Assuming that these protons are a suitable model for H-3′ in structure **4**, the value of δ 4.32 in the pigment would exclude this structure **4**. Structures analogous to **1**, **2**, **3**, and **4**, but with the acetate group at other hydroxyl positions, were eliminated on the basis of similar arguments.

It was expected that structures **5** and **6** could be distinguished from a determination of the number of *peri*-hydroxyl signals and their integrations. For reasons discussed later, this distinction was not possible. Because of the number of metabolites which preferentially form acetals similar to versicolorins (Hamasaki et al., 1967) (cyclization and ring closure at the carbon corresponding to C-6′) it seems reasonable that the analogous hemiacetal should also be preferred. (Structure **5** would require the unfavorable loss of an intramolecular hydrogen bond.) For this reason, structure **6** should be more likely than **5**.

^{13}C signals for C-1 and C-3 through C-14 in VHA were assigned to the carbon atoms forming the basic polyhydroxyanthraquinone structure by comparison with the carbon spectrum of averufin (structure **7**) (Fitzell et al., 1975; Gorst-Allman et al., 1976). As shown in Table I, exact assignments of these signals could not be made because of the similarity of the chemical shifts. The remaining seven signals were assigned on the basis of the following data.

In the spectra of VHA from [1-^{13}C] and [2-^{13}C], only two signals showed directly bonded carbon–carbon coupling (J_{C-C} = 42 Hz, sp^3–sp^2). These signals at δ 121.6 and 44.6 were assigned to C-2 and C-5′, respectively. Carbon-2, which forms the bridge between the incomplete bisfuran structure and the rest of the molecule, was assigned to the signal at δ 121.6 because it was the only remaining unassigned signal in the aromatic region and because of its sensitivity to temperature changes (see below).

The signal at δ 44.6 was assigned to C-5′ because of the previously mentioned coupling, its multiplicity (d) under off-resonance proton-decoupled conditions and its chemical shift. Both the chemical shifts and multiplicities of the C-2 and C-5′ signals were temperature and solvent dependent. This would be expected for cis and trans hemiacetals (the hydroxyl group of C-6′ being cis or trans to the proton on C-5′) that are interconverting at a rate comparable to the NMR time scale.

The signal at δ 116.2 was assigned to C-6′ on the basis of its chemical shift which was similar to that found in the acetal carbon of aflatoxin B_1 (δ 113.6) (Hsieh et al., 1975). The C-6′ signal was broad in all spectra recorded, again indicating that the hemiacetal ring is interconverting between the two diastereomers.

The signal at δ 29.4, assigned to C-4′, appeared to be a superimposition of two or more signals as would be expected from the presence of two diastereomers. Its chemical shift also agrees well with similar aliphatic carbons (Levy and Nelson, 1972; Johnson and Jankowski, 1972, Spectrum 273).

The signal at δ 62.7 was assigned to C-3′. It appeared as a triplet in the off-resonance proton-decoupled spectrum, and its chemical shift is consistent with an acetate of a primary alcohol (Johnson and Jankowski, 1972, Spectrum 411).

The methyl carbon of the acetate (δ 20.7) was easily assigned because of its multiplicity (q) in the off-resonance proton-decoupled spectrum and its characteristic chemical shift (Johnson and Jankowski, 1972, Spectra 345, 411). The signal at δ 170.8 is consistent with a carbonyl in an acetate unit (Johnson and Jankowski, 1972, Spectra 345 and 411) and was coupled (J_{C-C} = 59 Hz) with the signal at δ 20.7 in the spectrum of VHA derived from dilabeled acetate.

Low sensitivity and poor resolution, attributed both to the presence of interconverting diastereomers and to the pigment's low solubility, made it impossible to determine the C–C coupling constants of many of the carbons in VHA prepared from dilabeled acetate.

Additional support of the proposed structure **6** was found in the ^{1}H NMR spectrum of the pigment (Table II). Our work basically agrees with earlier published reports (Yao and Hsieh, 1974; Schroeder et al., 1974) on the pigment and on model compounds in the literature (Thomas, 1971) with respect to the three aromatic protons of the molecule. However, in contrast to the meta coupling constants (4.0 Hz) reported by Schroeder et al. (1974), our work indicated a coupling of only 2.4 Hz.

The signal at δ 6.47 integrates for one proton and is a doublet with a spacing of ~2 Hz at room temperature. The doublet merges and becomes a single broad peak at −40 °C. The chemical shift and temperature dependence of this peak is consistent with that expected for two exchanging diastereomeric protons (H-6′). The corresponding acetal proton in the trimethyl ether of versicolorin A (Thomas, 1971) is found at δ 6.72.

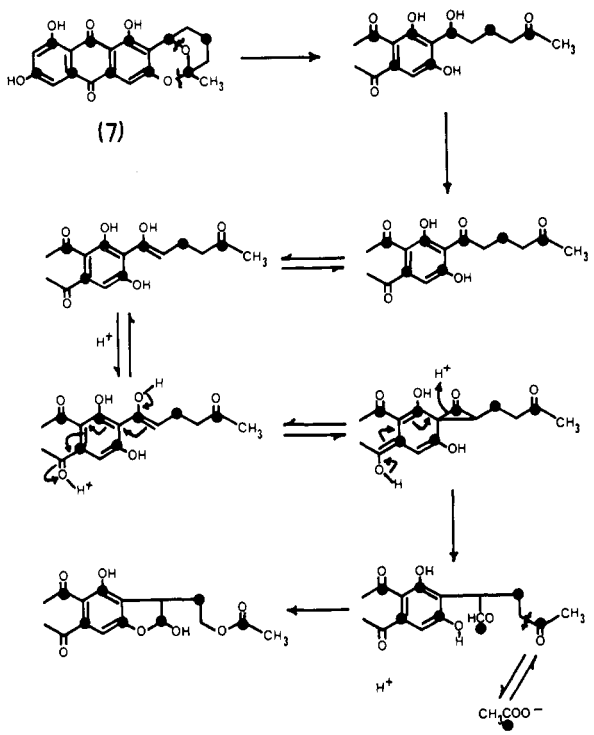

Figure 2. The proposed scheme for the conversion of the C_6 side chain of averufin into the side chain of versiconal hemiacetal acetate.

A broad peak centered at δ 12.1 at room temperature separates into two peaks at low temperature (δ 12.24 and 12.56 at −50 °C) and integrates for a total of ca. one proton at each temperature. This signal is assigned to the *peri*-hydroxyl protons (H_A, H_B). These shifts are consistent with those found for the *peri*-hydroxyl protons in 1,6,8-trihydroxy-3-methylanthraquinone (emodin), δ 12.16 and 12.03 (Table II). Of considerable concern was the observation that these peaks integrated to a total of ca. one proton which suggests the possibility of structure **5**. However, under similar conditions (in acetone-d_6) integration for only one *peri*-hydroxyl proton was obtained with 1,8-dihydroxyanthraquinone (in chloroform-d_1, 1,8-dihydroxyanthraquinone gives integrations corresponding to two *peri*-hydroxyl protons). In addition, numerous spectra of emodin were obtained where the number of *peri*-hydroxyl protons vary from 1.1 to ~2.

The variability of the *peri*-hydroxyl proton integrations in these compounds suggests that the hydrogen bond strengths in a di-*peri*-hydroxyl hydrogen bonded system may be considerably weaker than that for a mono-*peri*-hydroxyl hydrogen bonded system. This could result in the observation of only one intramolecular hydrogen bonded *peri*-hydroxyl group in solvent systems where there is intermolecular competition for hydrogen bonding or hydrogen exchange. In solvent systems where the external competition is lower, one should then observe two *peri*-hydroxyl groups. Further investigation of this possibility is in progress.

An additional argument against structure **5** is that both *peri*-hydroxyl hydrogen peaks are of equal intensity in the pigment. This would require identical amounts of each hemiacetal diastereomer and this is unlikely on thermodynamic grounds.

Selective proton decoupling showed that the aromatic meta protons, H-5 and H-7, were coupled. The triplet assigned to H-3′ (2 H) at δ 4.32 (J = 6.6 Hz) has a chemical shift consistent with protons adjacent to an acetoxy group (Silverstein et al., 1974, appendix b) rather than an hydroxyl group. This triplet collapses to a singlet with irradiation at δ ~2.0, allowing assignment of the broad multiplet at δ ~2.0 to the H-4′ protons. The signal at δ 2.64 (1 H) is a broad multiplet consisting of at least eight lines at room temperature and was assigned to H-5′. The multiplicity of the signal is to be expected since these protons are a diastereomeric pair and are also coupled to diastereotopic H-4′ protons as well as to the diastereomeric H-6′ protons. The H-4′ protons are diastereomeric, diastereotopic, and also coupled to three other protons. As a result, selective decoupling of H-3′ did not simplify the H-4′ multiplet. In methanol-d_4 H-1′ was found at δ 2.1, and the total integration for H-4′ and H-1′ showed ca. five protons. Additional signals found in all ^1H spectra were attributed to impurities. These signals varied from sample to sample in their relative intensities. In addition, it was possible to isolate from the pigment by TLC a fraction that had a proton spectrum containing the additional signals.

The labeling pattern of VHA derived from [1-^{13}C]- and [2-^{13}C]acetate agrees with that of aflatoxin B_1 and the other intermediates already investigated (Fitzell et al., 1975; Hsieh et al., 1975; Tanabe et al., 1970) and is shown in structure **6**.

The use of both [1-^{13}C]- and [2-^{13}C]acetate as precursors in this study clearly shows that acetate is the sole carbon source of this molecule. Because VHA was found to be an intermediate between averufin and aflatoxin B_1 (Yao and Hsieh, 1974; Singh and Hsieh, 1977), support is given to earlier findings (Hsieh et al., 1976) that the bisfuran ring system of aflatoxin B_1 is formed from the six-carbon side chain of averufin.

Still of major importance in the biosynthetic pathway of aflatoxins is the mechanism through which the bisfuran ring structure is formed. Several schemes (Moss, 1972; Roberts, 1973; Kingston et al., 1976) have been proposed, none of which accounts for a biosynthetic intermediate with the structure of VHA. Therefore, a scheme is proposed which accounts for VHA as a biosynthetic intermediate (Figure 2). Of particular interest is the last step in the proposed scheme. We are proposing the removal of the two terminal carbons, followed by the addition of an acetyl group from labeled acetate still present in the medium. If this step occurs prior to ring closure and formation of the hemiacetal, an intermediate corresponding to that proposed by Schroeder et al. (1974) could be formed. (Schroeder et al., (1974) also considered the possibility of the acetate group being attached to the side chain.) Possible differences in experimental procedure could account for the different results. Our laboratory is presently attempting to convert labeled averufin into VHA in an unlabeled medium in order to determine the origin of the acetate unit in VHA. A significantly lower level of labeling in this acetate group is consistent with our proposed last step. This scheme is purely speculative since no intermediates between averufin and VHA have been determined, but it does account for the unique labeling pattern found in aflatoxins and all biosynthetic data currently known.

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Paspalum Staggers: Isolation and Identification of Tremorgenic Metabolites from Sclerotia of *Claviceps paspali*

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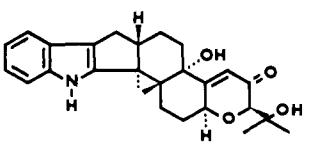
The neurological disorder, Dallisgrass poisoning or paspalum staggers, occurs in cattle that graze *Paspalum dilatatum* infected with the fungus *Claviceps paspali* and occurs sporadically in the southern portions of the U.S. Three tremorgenic metabolites have been isolated from *C. paspali* sclerotia collected from *Paspalum dilatatum*. The identification of these metabolites has been accomplished by spectroscopic methods. One of the metabolites is identical with paspalinine, a previously reported metabolite of *C. paspali*. The remaining two metabolites differ from paspalinine in that they contain an additional isoprene and hydroxyisoprene unit attached to carbon 5 of the six-membered indole ring, 3-methyl-2-butenylpaspalinine and 3-hydroxy-3-methyl-1-butenylpaspalinine, respectively.

A naturally occurring neurological disorder, Dallisgrass poisoning, also called "paspalum staggers", occurs when cattle graze *Paspalum dilatatum* infected with the fungus, *Claviceps paspali* (Brown, 1916). *C. paspali* invades the pistil of the grass flower and destroys the ovary, which is replaced by a mass of fungal tissue. Spores are produced in great abundance along with a sticky, sweetish exudate of the fungal tissue termed "honeydew". Insects attracted to the exudate and cattle or other animals walking through the grass spread the infection to other grass plants. If conditions are favorable, the fungus forms a sclerotium which is toxic to grazing cattle (Grayson, 1941). Other microorganisms, notably *Fusarium heterosporum*, may colonize the honeydew, ultimately inhibiting sclerotium maturation (Cunfer, 1975). Immature sclerotia are smaller in size and appear orange in color.

Clinical signs of "paspalum staggers" are tremors which are exaggerated by enforced movement, hyperexcitability, and ataxia. Mortalities from the disease are generally caused by accident or inability of affected animals to obtain water. Affected animals generally recover from the disease if removed from the toxic pasture.

Paspalum staggers occurs sporadically in the southern portions of the U.S. where the host plant is found. In Louisiana, the disease was more extensive in 1976 than in the three previous years. The increased incidence of the disease was associated with lower than normal rainfall in most parts of the state.

Prompted by the availability of field-produced sclerotia and the observation that paxilline (I), a tremorgenic



I

metabolite of *Penicillium paxilli* (Cole et al., 1974; Springer et al., 1975), was closely related to previously reported metabolites [paspaline (II), paspalicine (III), and

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