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Toxins Causing Noninflammatory Paralytic Neuronopathy. Isolation and Structure Elucidation

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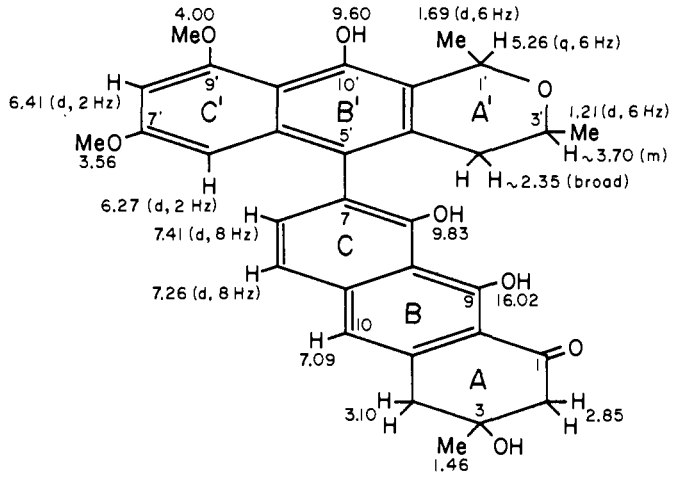
Abstract: Four constituents of the neurotoxic principal of *Karwinskia humboldtiana* (Rhamnaceae) have been isolated and identified as 7-[3′,4′-dihydro-7′,9′-dimethoxy-1′,3′-dimethyl-10′-hydroxy-1′*H*-naphtho[2′,3′-*c*′]pyran-5′-yl]-3,4-dihydro-3-methyl-3,8,9-trihydroxy-1(2*H*)-anthracenone (**1**), 3,4-dihydro-3,3′-dimethyl-1′,3,8,8′,9-pentahydroxy(7,10′-bianthracene)-1,9′(2*H*,10′*H*)-dione (**2**), 7-(2′-acetyl-6′,8′-dimethoxy-3′-methyl-1′-hydroxynaphth-4′-yl)-3,4-dihydro-3-methyl-3,8,9-trihydroxy-1(2*H*)-anthracenone (**3**), and 3,3′-dimethyl-3,3′,8,8′,9,9′-hexahydroxy-3,3′,4,4′-tetrahydro(7,10′-bianthracene)-1,1′(2*H*,2′*H*)-dione (**4**). In addition, occurrence of the C-7′ desmethoxy analog of **1** was indicated indirectly and chrysophanol (**5**), 3,4-dihydro-7,9-dimethoxy-1,3-dimethyl-1*H*-naphtho[2,3-*c*]-5,10-pyrandione (7-methoxyelutherin, **6**), 3,4-dihydro-7,9-dimethoxy-1,3-dimethyl-6-hydroxy-1*H*-naphtho[2,3-*c*]pyran-5,10-dione (**7**), and 2-aceto-6,8-dimethoxy-3-methyl-1-naphthol (tarachrysone monomethyl ether, **8**) were isolated from a nontoxic hexane extract of *K. humboldtiana* seeds. Proton magnetic resonance spin decoupling experiments using **6** established that the C-1 and C-3 methyl groups in this compound are cis diequatorial; by analogy it is assumed that the stereochemistries of the dihydrodimethylpyran rings of **1** and **7** are similar.

Fractionation of the neurotoxic principle of *Karwinskia humboldtiana*, Zucc. (Rhamnaceae) has led to the isolation of four major constituents to which structures **1–4** (Figure 1) have been assigned. Compounds **1–4**, comprise respectively 0.75, 0.59, 0.39, and 0.46% of the weight of air-dried mature fruit and seeds and account for the observed neurotoxic properties.[2]

Clavigero,[3] almost 2 centuries ago, first reported the toxic effects which result from ingestion of the seeds[4,5] of *K. humboldtiana*, a plant indigenous to desert areas of southern Texas and northern and central Mexico. Clinically the toxicity is characterized by a progressive and symmetrical noninflammatory paralytic neuronopathy starting in the lower limbs and ending with respiratory and bulbar paralysis.[6]

Isolation of Toxins. The yellow, amorphous crude toxin,[4] obtained by chloroform extraction of the toxic seeds, was fractionated by chromatography on silica gel; compound **2** was eluted using benzene–acetone (50:1) with elution of the other, more polar constituents requiring increased acetone concentrations, i.e., **1** (20:1), **3** (10:1), and **4** (5:1). Pure samples of each of the toxins were obtained only after repeated rechromatography of these initial fractions. Fractionation of hexane soluble material from *K. humboldtiana*



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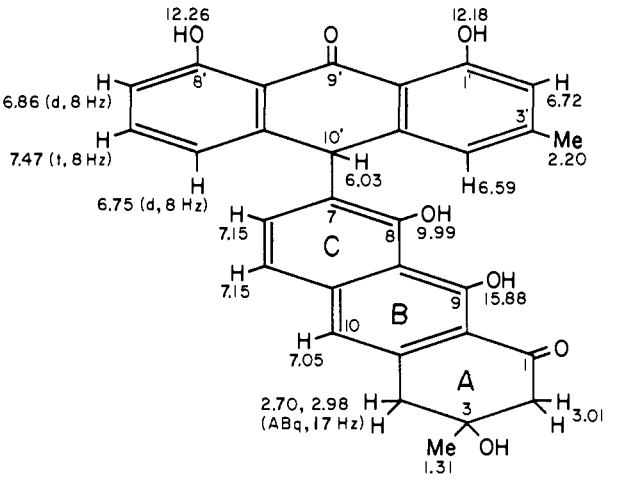

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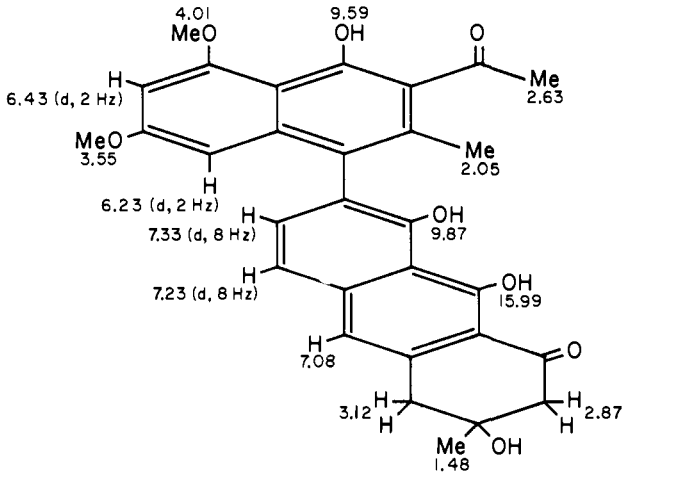

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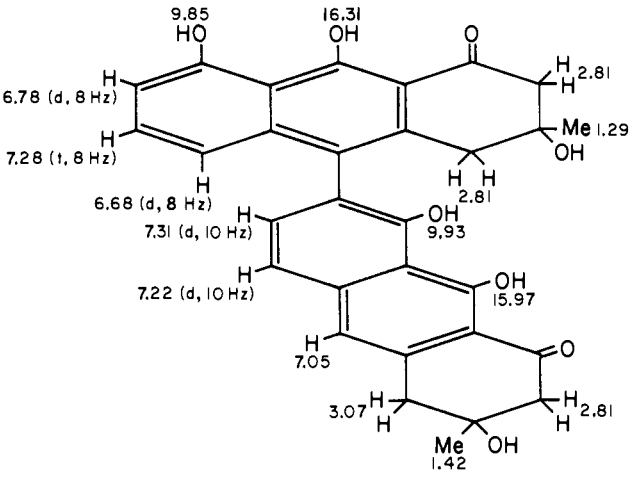

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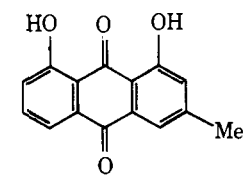
Figure 1. Structures and proton magnetic resonance (^{1}H NMR) data for constituents of *K. humboldtiana* toxic principle. ^{1}H NMR data for 1, 3, and 4 were obtained using deuteriochloroform solutions; for **2** a dimethyl-d_6 sulfoxide solution was used. Assignments of resonances are based on extensive spin decoupling experiments (e.g., in the spectra of **2** and 4 irradiation of the easily identified meta hydrogen resonances (C-6′ H) allowed resonances for C-5′ H and C-7′ H to be distinguished from those of ring C-hydrogens) and utilized spectra obtained in a variety of solvents (e.g., in dimethyl-d_6 sulfoxide–deuterium oxide the aromatic protons resonances for **4** were well resolved and facilitated assignment).

seeds (which is nontoxic)[4] yielded chrysophanol (**5**) as previously noted by Dominguez and Garza[7] and, in addition, 3,4-dihydro-7,9-dimethoxy-1,3-dimethyl-1*H*-naphtho[2,3-*c*]-5,10-pyrandione (7-methoxyelutherin, **6**), 3,4-dihydro-7,9-dimethoxy-1,3-dimethyl-6-hydroxy-1*H*-naphtho[2,3-*c*]pyran-5,10-dione (**7**), and 2-aceto-6,8-dimethoxy-3-methyl-1-naphthol (tarachrysone monomethyl ether, **8**).[8,9]



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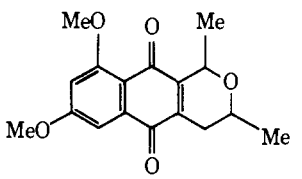

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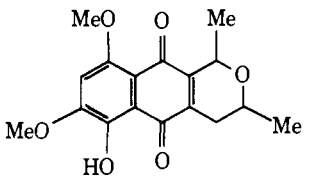

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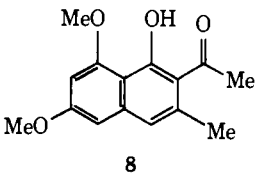

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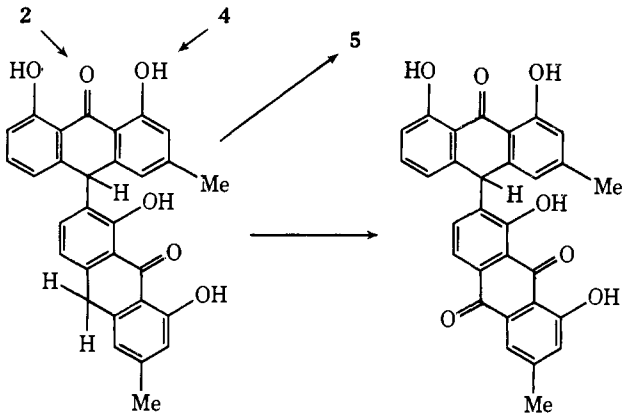
Assignment of Toxin Structures. High resolution mass spectrometry established the elemental compositions of the four major components of the toxic principle (i.e., **1**, $C_{32}H_{32}O_8$;[4b,c] **2**, $C_{30}H_{24}O_7$; **3**, $C_{30}H_{28}O_8$; **4**, $C_{30}H_{26}O_8$), gave indication of their "dimeric" nature, and provided evidence for a common structural feature for each of the compounds. In the mass spectrum of each of compounds **1–4** a prominent ion was observed at *m/e* 240 ($C_{15}H_{12}O_3$); in the spectrum of **1** an ion at *m/e* 286 ($C_{17}H_{18}O_4$) corresponding to the other "monomer" unit in this compound was also observed. The ultraviolet-visible spectra (Table I) show multiple, intense absorptions in the region 220–270 nm with weaker long wavelength bands at 400–420 nm, and the infrared spectra (Table I) show hydrogen bonded carbonyl bands at 1600–1635 cm^{-1}. These data show close similarities to those of known 3,4-dihydro-8,9-dihydroxy-1(2*H*)-anthracenones[10–20] and suggest this structural unit as a feature common to each of toxins **1–4**.

Definitive structural assignments resulted from detailed analyses of the proton magnetic resonance (^{1}H NMR) spectra of the toxins (see Figure 1), certain transformation products, and the related lower molecular weight constituents (**5–8**) also present in *K. humboldtiana* extracts. Characteristic resonances which identify the 3,4-dihydro-3-methyl-3,8,9-trihydroxy-1(2*H*)-anthracenone system (i.e., ring system ABC)[10–12,19] present in each of compounds **1–4** are: (a) hydrogen bonded phenolic hydroxyl resonances near δ 10 and 16, (b) two two-proton singlets in the region δ 2.75–3.20 assigned to C-2 and C-4 protons, (c) a singlet methyl resonance (C-3 methyl) near δ 1.5, and (d) a one proton singlet near δ 7.1 assigned to H-10.

Toxin 4. That compound **4** is an unsymmetrical dimer of this dihydroanthracenone system was evident from the presence, in each of spectral regions a, b, c noted above, of similar, but not always superimposed, resonances corresponding to two sets of identical structural features in slightly differing environments. Significantly, only a single resonance is present near δ 7.1 (see point d above) indicating that one terminus of the junction between the two tetrahydroanthracene systems is C-10′. Confirmation of this assignment was obtained when **4** was didehydrated and oxidative cleavage[21] of the resulting anhydro derivative (**9**) yielded chrysophanol (**5**).[7] That the other terminus is C-7 is evident from analysis of the ^{1}H NMR spectrum of the anthraquinone (**10**) ob-



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tained by air oxidation of **9**. In this spectrum the signal (δ 7.68, d, J = 10 Hz) assigned[22] to H-5 is 0.5 ppm downfield of the corresponding resonance in the spectrum of **4**; this downfield shift of a peri proton resonance in going from an anthracene to the corresponding anthraquinone is highly characteristic.[23]

Toxin 2. The relationship between compounds **2** and **4**, and therefore the structure of **2**, was evident when **9** was also obtained by dehydration of **2**.

Toxin 1. The second "monomer" unit of **1** was identified as ring system A′B′C′ (closely related to elutherin isolated from tubers of *Eleutherine bulbosa*, Iridaceae[24]). Recognition and characterization of this system, and particularly the dihydrodimethylpyran portion (ring A′), was greatly facilitated by earlier, extensive ^{1}H NMR investigations of similar systems by Cameron and Todd[25] in connection with investigations of aphid pigments. ^{1}H NMR resonances observed in the spectrum of **1**[26](see Figure 1) which are associated with the methoxyeleutherin system include a phenolic hydroxyl resonance at δ 9.60, aromatic proton resonances at δ 6.27 and 6.41, a methine quartet at δ 5.26, methoxyl proton singlets at δ 3.56 and 4.00, and C-methyl doublets at δ 1.69 and 1.21. Assignment of the methoxyl resonances to ring C′ and the aromatic proton resonances which show meta coupling (J = 2 Hz) to H-6′ and H-8′ of this ring is

Table I. Ultraviolet and Infrared Data for *K. humboldtiana* Toxin Constituents and Transformation Products

Compound	Uv λ_{max}^{MeOH}, nm (log E)	Ir ν_{max} (KBr), cm^{-1}
1	228 (4.67), 242 (4.77), 270 (4.63), 415 (4.00)	3390, 1625
2	227 (4.66), 273 (4.71) 397 (4.15)	3415, 1635, 1620, 1600
3	228 (4.72), 238[a] (4.69), 268 (4.67), 410 (3.99)	3390, 1625
4	227 (4.64), 269 (4.73), 410 (4.13)	3390, 1625
9	260 (4.32), 280 (4.17), 373 (4.26)	3415, 1640, 1620, 1600
10	227 (4.81), 261 (4.65), 287 (4.38), 390 (4.30), 430 (4.28)	3435, 1640, 1620, 1600
11	238 (4.88), 296 (4.30), 345 (4.28)	3390, 1620, 1605
12	228 (4.92), 240 (4.88), 290 (4.26), 430 (4.18)	3390, 1625, 1605
13	228 (5.00), 255[a] (4.46), 290 (4.23), 430 (4.17)	3390, 1625, 1605

[a] Shoulder.

supported by the formation of 3,5-dimethoxyphthalic anhydride[27] upon permanganate oxidation of **1** and by the observation of similar resonances in the spectra of simpler, structurally related compounds, **6–8** (see Experimental Section). The ^{1}H NMR resonances which characterize ring A′ are the following: an A_3X system (C-1′ H, quartet at δ 5.27 and C-1′ methyl, doublet at δ 1.69) and a more complex system consisting of the C-3′ methyl (doublet at δ 1.21) and C-3′ H and C-4′ H's (unresolved resonances at δ 3.70 and 2.35, respectively). The indicated spin–spin interactions were established, in each case, by appropriate decoupling experiments. The presence of a phenolic hydroxyl resonance and the absence of an aromatic resonance assignable to C-5′ H rules out the keto tautomer for the C-10′ oxygen function and establishes C-5′ as the linkage point to ring system ABC. That this linkage is to C-7 was established as in the case of toxins **2** and **4**; i.e., comparison of chemical shifts for the C-5 H resonances of **1** (δ 7.41) and anthraquinone **12** (δ 7.97), prepared by oxidation of anhydro derivative **11**, shows the expected downfield shift owing to the presence in **12** of the carbonyl at C-10.[23]

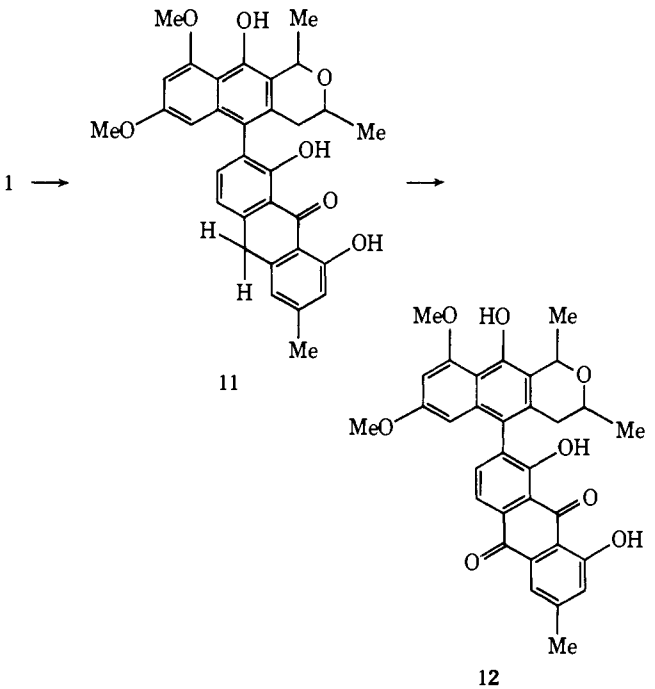

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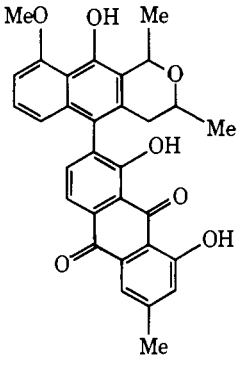
Table II. ^{1}H NMR Chemical Shifts and Coupling Constants for 3,4-Dihydro-7,9-dimethoxy-1,3-dimethyl-5,10-naphtho[2,3-*c*]-pyrandione (**6**) Which Determine the Stereochemistry of the Dihydrodimethylpyran Ring

Hydrogen	Chemical shift $\delta(CDCl_3)$, ppm	Coupling constant, Hz
C-1 Me(e')[a]	1.52	$J_{Me,C\text{-}1H} = 6$[b]
C-3 Me(e')	1.34	$J_{Me,C\text{-}3H} = 6$
C-1 H(a')	4.48	$J_{C\text{-}1a,C\text{-}4a} = 4$
		$J_{C\text{-}1a,C\text{-}4e} = 2$
C-3(a')	3.58	$J_{C\text{-}3a,C\text{-}4a} = 10$
		$J_{C\text{-}3a,C\text{-}4e} = 2$
C-4(a')	2.18	$J_{C\text{-}4a,C\text{-}4e} = 18$
C-4(e')	2.71	

[a] e', pseudoequatorial; a', pseudoaxial. [b] Obtained by irradiation of signals at δ 4.48 and 3.58.

Chromatographic purification of **12**, prepared from a sample of **1** only partially purified, resulted in the isolation of a minor component which was assigned structure **13**, i.e.,



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the 7'-desmethoxy analog of **12**, on the basis of its mass and ^{1}H NMR spectra. The methoxyl group of **13** is assigned to C-9' rather than C-7' because its ^{1}H NMR chemical shift (δ 4.00) is that expected;[28] presumably the high field methoxyl resonances observed in the spectra of **1**, **11**, and **12** are the result of diamagnetic shielding of the C-7' methoxyl group by aromatic ring C in these "dimers".

The isolation of **13** strongly implies the presence in the original *K. humboldtiana* extract of the C-7' desmethoxy analog of **1** although repeated attempts to detect this compound chromatographically were unsuccessful. While mass spectrometry is often useful for the detection of trace constituents, it is not useful in the present instance since loss of formaldehyde from **1** or its transformation products upon electron bombardment (or thermally in the mass spectrometer inlet system) is expected to be facile.[29,30]

Toxin 3. The remaining ^{1}H NMR resonances in the spectrum of **3** (Figure 1) following assignment to the trihydroxyanthracenone (ABC) system show certain features in common with those of toxin **1**. Thus, as in the spectrum of **1**, resonances assignable to two methoxyl groups and two meta disposed aromatic hydrogens are indicative that ring C' of the two toxins is common. Unlike **1**, however, the spectrum of **3** lacked the high field C-methyl resonances characteristic of a dihydrodimethylpyran ring (A' in structure **1**); instead two C-methyl resonances were observed at δ 2.05 and 2.63 assignable as indicated (Figure 1). The isolation of the known "monomer" **8**[8,9] from a nonpolar extract of *K. humboldtiana* further supports the assigned structure.

Constituents of Nonpolar, Nontoxic Extract. Although it has previously been shown[4] that the hexane soluble material from seeds of *K. humboldtiana* is nontoxic, compounds **5–8** isolated from this extract are closely related in structure to the constituents (**1–4**) of the toxic principle. Chrysophanol (**5**) previously isolated from *K. humboldtiana*[7] and 2-aceto-6,8-dimethoxy-3-methyl-1-naphthol (tarachrysone monomethyl ether, **8**)[8,9] were identified by comparison with authentic samples.[31] Assignment of structures **6** and **7** was straightforward upon consideration of their spectra (see Experimental Section).

Stereochemical Assignments. Detailed analysis of the ^{1}H NMR spectrum of **6** revealed the relative stereochemistry of substituents of the dihydrodimethylpyran ring. With the aid of spin-spin decoupling experiments all coupling constants were obtained (Table II) and the resulting analysis, which follows closely those of Cameron and Todd,[25] indicates that both C-1 H and C-3 H are pseudoaxial. Thus, the observation of a large coupling constant (J = 10 Hz) for C-3 H and the C-4 axial hydrogen requires trans diaxial stereochemistry.[32] Similarly, the magnitude (4 Hz) of the long range (five bond) coupling constant for interaction between C-1 H and the C-4 axial hydrogen requires that C-1 H be pseudoaxial.[25,32] Therefore, the C-1 and C-3 methyl groups of **6** are cis diequatorial, i.e., the stereochemistry observed for eleutherin[24,25] the C-7 desmethoxy analog of **6**, and not that of the related trans dimethyl isomer, isoeleutherin.[24,25] By analogy, it is assumed that the corresponding methyl groups of the dihydrodimethylpyran rings of **1** and **7** (and the transformation products **11**, **12**, and **13**) are also cis diequatorially disposed.

Experimental Section

Melting point determinations were made with a Thomas hot-stage. ^{1}H NMR spectra were obtained using a Varian HA-100 spectrometer, ultraviolet spectra were recorded on a Cary 15 or Perkin-Elmer 202, infrared spectra were recorded on a Perkin-Elmer 337, and mass spectra were measured using a CEC 21-110B high resolution mass spectrometer. Thin layer chromatography (TLC) was performed on silica gel G plates.

Isolation of Toxic Principle.[4] Air-dried mature fruit of *K. humboldtiana* (755 g) was finely ground, suspended in 800 ml of chloroform, and shaken mechanically for 12 hr. The residue was removed by filtration and the process was repeated twice more. The combined chloroform extracts were then concentrated to a volume of ~150 ml; this solution was poured into 2-l. of rapidly stirring *n*-hexane. The resulting precipitate was collected, the precipitate was redissolved in chloroform, and the precipitation procedure was repeated yielding 19.5 g of the toxic principle as a dull yellow powder.

Fractionation of Toxic Principle.[4] A 1-g portion of the toxic principle was chromatographed on 100 g of silicic acid (column 2 × 70 cm) using benzene and acetone for elution. Compounds **1–4** were eluted as discrete colored bands. Elution with benzene–acetone (50:1) yielded **2** (0.23 g); benzene–acetone (20:1) eluted **1** (0.29 g), **4** (0.15 g) eluted with benzene–acetone (10:1), and **3** (0.18 g) was produced using benzene–acetone (5:1). Compounds **1–4** were purified for characterization as described below.

7-[3',4'-Dihydro-7',9'-dimethoxy-1',3'-dimethyl-10'-hydroxy-1'*H*-naphtho[2',3'-*c*']pyran-5'-yl]-3,4-dihydro-3-methyl-3,8,9-trihydroxy-1(2*H*)-anthracenone (1). The material obtained as above was rechromatographed five times as described and then recrystallized from a benzene–hexane mixture to yield **1**: mp 166–168°; uv and ir data are in Table I; ^{1}H NMR data[26] are in Figure 1; MS M^+ 544.2083 (calcd for $C_{32}H_{32}O_8$, 544.2098).

3,4-Dihydro-3,3'-dimethyl-1',3,8,8',9-pentahydroxy(7,10'-bianthracene)-1,9'(2*H*,10'*H*)-dione (2). The product obtained from chromatography (see above) was recrystallized from chloroform–hexane to produce **2** as yellow needles: mp 230° dec; uv and ir data are recorded in Table I; ^{1}H NMR spectra are in Figure 1. MS, M^+ 496.1506 (calcd for $C_{30}H_{24}O_7$, 496.1522).

7-(2'-Aceto-6',8'-dimethoxy-3'-methyl-1'-hydroxynaphth-4'-yl)-3,4-dihydro-3-methyl-3,8,9-trihydroxy-1(2*H*)-anthracenone (3). Repeated (five times) chromatography using the system described for the initial separation followed by recrystallization from chloroform–hexane gave **3**: mp 146–148°; uv and ir data are in Table I;

^{1}H NMR data are in Figure 1; MS, M^+ 516.1768 (calcd for $C_{30}H_{28}O_8$, 516.1784).

3,3′-Dimethyl-3,3′,8,8′,9,9′-hexahydroxy-3,3′,4,4′-tetrahydro-(7,10′-bianthracene)-1,1′(2*H*,2′*H*)-dione (4). The crude compound obtained by fractionation of the toxic principle (see above) was rechromatographed five times under similar conditions and then recrystallized from chloroform–hexane to yield **4** as a yellow powder: mp 130° dec; see Table I for uv and ir data and Figure 1 for ^{1}H NMR data; MS, M^+ 514.1634 (calcd for $C_{30}H_{26}O_8$, 514.1628).

3,3′-Dimethyl-1,1′,8,8′-tetrahydroxy(7,10′-bianthracene)-9,9′(10*H*,10′*H*)-dione (9), Method A. A solution of 200 mg of **2** in 50 ml of pyridine under nitrogen was stirred at room temperature for 1 hr. The solution was then poured into 6 *N* hydrochloric acid and extracted with chloroform. The chloroform extract was washed with water, dried, and evaporated to yield, after recrystallization from benzene, 190 mg (98%) of **9**: mp 240° dec; uv and ir data are in Table I; ^{1}H NMR ($CDCl_3$), δ 2.23, 2.34 (6 H, s, C-3, C-3′ methyls), 4.20 (2 H, s, C-10 H's), 6.06 (1 H, s, C-10′ H), 12.12, 12.27, 12.37, 12.90 (4 H, s, C-1, C-1′, C-8, C-8′ OH's).

Method B. A solution of 100 mg of **2** in 10 ml of acetic acid containing 10 drops of concentrated hydrochloric acid was heated on a steam bath for 5 hr. Water was added until precipitation occurred; the product was filtered and recrystallized from benzene to yield 73 mg (76%) of **9**.

Method C. Dehydration of 56 mg of **4** using acetic acid (method B) followed by silicic acid chromatography with benzene–acetone (20:1) yielded 18 mg (34%) of **9** and 14 mg (26%) of a monodehydration product (MS, *m/e* 496, M^+).

7-[3′,4′-Dihydro-7′,9′-dimethoxy-1′,3′-dimethyl-10′-hydroxy-1′*H*-naphtho[2′,3′-*c*′]pyran-5′-yl]-1,8-dihydroxy-3-methyl-9-anthracenone (11). Dehydration of 500 mg of **1** in pyridine as described (see **9**, method A) yielded, after chromatography on silicic acid using chloroform and recrystallization from methanol, 408 mg (84%) of **11**: mp 165–167°. (For this dehydration acetic acid (see above) proved unsatisfactory.) Table I contains uv and ir data for **11**; ^{1}H NMR ($CDCl_3$),[26] δ 1.27 (3 H, d, C-3′ methyl), 1.67 (3 H, d, C-1′ methyl), 2.39 (3 H, s, C-3 methyl), 3.61 (3 H, C-7′ OCH_3), 4.02 (3 H, C-9′ OCH_3), 4.39 (2 H, s, C-10 H's), 5.39 (1 H, q, C-1′ H), 6.23 (1 H, d, C-6′ H), 6.43 (1 H, d, C-8′ H), 6.76 (2 H, s, C-2, C-4 H's), 7.02 (1 H, d, C-5 H), 7.32 (1 H, d, C-6 H), 9.53 (1 H, s, C-10′ OH), 12.21, 12.58 (2 H, s, C-1, C-8 OH's).

3,3′-Dimethyl-1,1′,8,8′-tetrahydroxy-7,10′-bianthracene-9,9′,10(10′*H*)-trione (10). A solution of 50 mg of **9** in 500 ml of methanol containing 5 ml of 1 *N* sodium bicarbonate was stirred for 20 min while a stream of air was passed through the solution. The solution was acidified using 6 *N* hydrochloric acid, the methanol was distilled, and the aqueous residue was extracted with chloroform. The chloroform extract was washed with water, dried, and evaporated. Chromatography of the resulting residue on silicic acid using benzene gave, after recrystallization from chloroform–hexane, 14 mg (27%) of **10** as small yellow-orange needles: mp 242° dec; uv and ir data are in Table I; ^{1}H NMR ($CDCl_3$), δ 2.24, 2.45 (6 H, s, C-3, C-3′ methyls), 6.03 (1 H, s, C-10′ H), 6.60, 6.74 (2 H, C-2′, C-4′ H's), 6.79, 6.90 (2 H, d's, C-5′, C-7′ H's), 7.11, 7.65 (2 H, C-2, C-4 H's), 7.38 (1 H, t, C-6′ H), 7.14 (1 H, d, C-6 H), 7.68 (1 H, d, C-5 H), 12.31, 12.41, 12.70, 12.93 (4 H, s, C-1, C-1′, C-8, C-8′ OH's).

7-[3′,4′-Dihydro-7′,9′-dimethoxy-1′,3′-dimethyl-10′-hydroxy-1′*H*-naphtho[2′,3′-*c*′]pyran-5′-yl]-1,8-dihydroxy-3-methylanthraquinone (12). A solution of 100 mg of **11** in 100 ml of methanol containing 5 ml of 1 *N* sodium bicarbonate was stirred for 1 hr while a stream of air was passed through the solution. Isolation of the product from the reaction mixture was accomplished as described (see **10**). Chromatography on silicic acid using dichloromethane and crystallization from methanol yielded 84 mg (82%) of **12** as orange brown needles: mp 176–178°; uv and ir data are in Table I; ^{1}H NMR ($CDCl_3$),[26] δ 1.23 (3 H, d, C-3′ methyl), 1.67 (3 H, d, C-1′ methyl), 2.47 (3 H, s, C-3 methyl), 3.60 (3 H, s, C-7′ OMe), 4.02 (3 H, s, C-9′ OMe), 5.25 (1 H, q, C-1′ H), 6.14, 6.43 (2 H, d's, C-5′, C-7′ H's), 7.10 (1 H, C-2 H), 7.64 (1 H, d, C-6 H), 7.69 (1 H, C-4 H), 7.97 (1 H, d, C-5 H), 9.65 (1 H, s, C-10′ OH), 11.96, 12.33 (2 H, s, C-1, C-8 OH's).

7-[3′,4′-Dihydro-1′,3′-dimethyl-10′-hydroxy-9′-methoxy-1′*H*-naphtho[2′,3′-*c*′]pyran-5′-yl]-1,8-dihydroxy-3-methylanthraquinone (13). When 500 mg of **1** as obtained by initial chromatography of the toxic principle (see above) was dehydrated to **11** using pyridine (see above) and this material, without chromatographic purification, was oxidized to **12** as described, a minor component was observed by thin layer chromatography. By multiple (five times) chromatography on silicic acid using benzene was isolated 50 mg of **13**: mp 180–181°; uv and ir data are in Table I; MS, *m/e* 510 (M^+); ^{1}H NMR ($CDCl_3$),[26] δ 1.24 (3 H, d, C-3′ methyl), 1.70 (3 H, d, C-1′ methyl), 2.49 (3 H, s, C-3 methyl), 4.07 (3 H, s, C-9′ OMe), 9.88 (1 H, s, C-10′ OH), 12.01, 12.41 (2 H, s, C-1, C-8 OH's).

Oxidative Cleavage of 9 to Chrysophanol (5).[34] Air was passed through a solution of 10 mg of **9** in 5 ml of pyridine (room temperature) for 30 min. The solvent was evaporated, the residue was dissolved in chloroform, and the chloroform solution was washed with dilute hydrochloric acid, water, and then dried. Removal of the chloroform left a residue which, upon sublimation, yielded chrysophanol (**5**).[33]

Oxidation of 1 to 3,5-Dimethoxyphthalic Anhydride.[27] A solution of 100 mg of **1** in 25 ml of 1 *N* sodium hydroxide containing excess potassium permanganate was allowed to stand at room temperature overnight. A solution of sodium bisulfite was then added to consume excess permanganate and the solution was acidified and extracted with ether. After drying the ether solution was evaporated and the residue sublimed to yield 3,5-dimethoxyphthalic anhydride identified by comparing MS and uv spectra with those of an authentic sample.[27]

Fractionation of Hexane Extract. The hexane soluble material (see above) was chromatographed in 1–3-g portions on silicic acid using dichloromethane–chloroform mixtures. Fractions were monitored by thin layer chromatography and combined as appropriate. Multiple rechromatography of selected fractions allowed isolation and characterization of five components. Chrysophanol (**5**),[7,33] β-amyrin,[7,34] and 2-aceto-6,8-dimethoxy-3-methyl-1-naphthol (**8**)[8,9,31] were identified by comparison with authentic samples. Previously unknown compounds were characterized by their spectrometric properties.

3,4-Dihydro-7,9-dimethoxy-1,3-dimethyl-5,10-naphtho[2,3-*c*]pyrandione (7-Methoxyeleutherin, 6). Following purification by repeated (five times) chromatography on silicic acid using dichloromethane, **6** was isolated as yellow needles exhibiting the following λ_{max}^{MeOH} 217, 264, 406 nm; ^{1}H NMR ($CDCl_3$), δ 3.95, 3.96 (6 H, s's, C-7, C-9 OMe), 6.69 (1 H, d, C-8 H), 7.22 (1 H, d, C-6 H), see Table II for other ^{1}H NMR data; MS, *m/e* 302 (M^+), 287 (M − Me).

3,4-Dihydro-7,9-dimethoxy-1,3-dimethyl-6-hydroxy-5,10-naphtho[2,3-*c*]pyrandione (7). This compound is bright red and exhibits the following: λ_{max}^{MeOH} 226, 279, 368, 494 nm; ^{1}H NMR ($CDCl_3$),. δ 1.31 (3 H, d, C-3 methyl), 1.46 (3 H, d, C-1 methyl), 2.23, 2.69 (2 H, m, C-4 H_2), 3.53 (1 H, m, C-3 H), 392, 395 (6 H, s's, C-7, C-9 OMe), 4.80 (1 H, m, C-1 H), 6.68 (1 H, s, C-8 H), 12.99 (1 H, s, C-6 OH); MS, *m/e* 318 (M^+).

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Purine Ring Rearrangements Leading to the Development of Cytokinin Activity. Mechanism of the Rearrangement of 3-Benzyladenine to N^6-Benzyladenine[1]

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Abstract: Under autoclaving conditions, certain 3-substituted adenines undergo rearrangement, in low conversion, to the corresponding N^6-substituted adenines which are cytokinins or plant growth substances. Using the 3-benzyladenine to N^6-benzyladenine conversion as illustration, we determined that the rearrangement was not due to a trivial sequence of N-3-α-C solvolysis followed by N^6-α-C alkylation under 2 atm of steam at 120° at pH 5.8. Rather, the rearrangement follows a contortional route involving ring opening and ring closing, during which the side chain does not leave its original nitrogen. In the process both rings open, the pyrimidine ring more readily than the imidazole ring. The solution to the problem of the route of rearrangement was based on the synthesis of specifically ^{15}N-labeled 3-benzyladenines and comparison of the products of their autoclaving, by mass spectrometry and NMR ^{15}N-^{1}H coupling, with N^6-benzyladenines specifically labeled with ^{15}N. Thereby, we determined that the products of autoclaving 3-benzyladenine-$^{15}N^6$ are N^6-benzyladenine, with label distributed about equally between N-3 and N-9, and 9-benzyladenine, with label distributed about equally between N-1 and N^6. Additional facts obtained in the course of this investigation establish the fragmentation pathways of substituted adenines in the mass spectrometer, provide diagnostic ^{15}N-^{1}H nuclear magnetic spin couplings for atom location, relate to the mutability of the adenine nucleus in a steam atmosphere, and suggest a chemical route for natural conversion to cytokinin-active substances.

Cytokinin is the generic name used to designate plant growth substances that play a major role in cell division and cell differentiation.[2-11] The measurement of cytokinin activity may be based on the growth of tobacco callus tissue, soybean callus tissue, or carrot tissue, the germination of seeds, or the retardation of leaf senescence. Cytokinin activity referred to in this paper will have been determined by the tobacco bioassay.[12,13] Whereas the existence of a specific cell division factor was postulated as early as 1892,[14] the modern era of research on the cytokinins began in 1955 with the isolation of kinetin, 6-furfurylaminopurine [N^6-(2-furfuryl)adenine], from old preparations of yeast DNA or from autoclaved, freshly prepared DNA.[15] The structure of the artefact was confirmed by synthesis, and this was quickly followed by the synthesis of other active analogs, especially N^6-substituted adenine derivatives and including 6-benzylaminopurine [N^6-benzyladenine or bzl^{6}Ade (**2**)].[16,17]

It has been established[18] that the cytokinin activity which develops when pure 1-benzyladenine (**1**) is autoclaved (pH 5.8, 120°, 2 atm of steam) either alone or in the plant growth medium is due to its conversion to 6-benzylaminopurine (**2**). The conversion pathway involves a 1-2 ring opening and 2-N^6 reclosure, as shown schematically by formulas **1a** → **2a** which identify the nitrogens, and it is representative of the general class of Dimroth rearrangements.[19-24] The autoclaving of 1-(Δ^2-isopentenyl)adenine similarly leads to the development of cytokinin activity due to its rearrangement to N^6-(Δ^2-isopentenyl)adenine (i^{6}Ade),[18] which may also be accomplished simply by refluxing in aqueous solution.

The task of establishing the reaction path or paths for the