AN INVESTIGATION OF THE ANTITUMOR ACTIVITY OF *SESBANIA DRUMMONDII*

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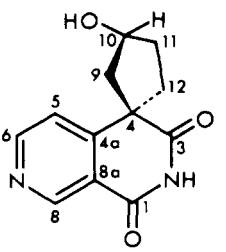
ABSTRACT.—Extracts of *Sesbania drummondii* (Rydb.) Cory seed are active *in vivo* in the P-388 lymphocytic leukemia system and cytotoxic in the KB assay. Systematic fractionation of *S. drummondii* has yielded sesbanine (**1**), a novel spirocyclic alkaloid based on the 2,7-naphthyridine nucleus, and drummondol (**2**), a new abscisic acid (**5**) metabolite. Sesbanine and drummondol are present in highly cytotoxic fractions; however, these purified compounds are essentially devoid of antitumor activity.

Sesbania drummondii (Rydb.) Cory (Fabaceae) is a perennial shrub or small tree inhabiting seasonally wet waste places and previously cultivated fields along the Coastal Plain from Florida to Texas. Seeds of *S. drummondii*, also known as coffeebean, rattlebrush, and rattlebox, are toxic to livestock, with the minimum lethal amount for sheep reported as about 0.1% of the animal's weight (1). In a previous report, we revealed that ethanolic extracts of the seed were cytotoxic



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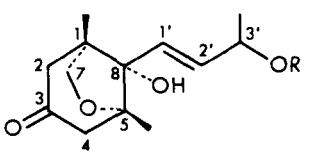
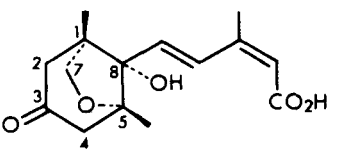


2, R = H
2a, R = C_7H_4OBr



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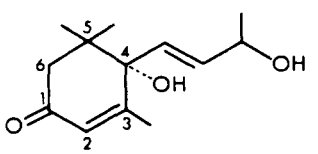

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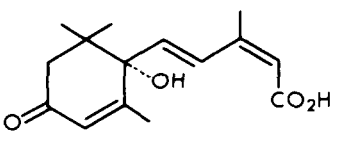

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[1]The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

in the KB cell culture and gave marked inhibition of P-388 lymphocytic leukemia (PS) in mice (2). Large-scale systematic fractionation of *S. drummondii* has revealed two new compounds, sesbanine (**1**) and drummondol (**2**). Although present in highly cytotoxic concentrates, **1** and **2** are essentially inactive after purification by hplc. We now report details of the isolation and characterization of these novel compounds.

An ethanol extract obtained from 454 kg of *S. drummondii* seed gave significant PS activity (T/C 175% at 45 mg/kg). Partition of the residue between aqueous ethanol and petroleum ether concentrated activity in the aqueous layer, and repeated washing of the aqueous layer with 1-butanol gave further concentration of activity in the butanol layer (T/C 153% at 10 mg/kg). Butanol solubles, 6.7 kg, were further fractionated by countercurrent distribution (ccd) using a three component biphasic system of water, ethyl acetate, and methanol in a 2:2:1 ratio. A preliminary 10-tube ccd experiment using 250 ml (125 ml each phase) of solvent per tube with 27 g of butanol solubles is summarized in table 1.

TABLE 1. Cytotoxic (KB) and antileukemic (PS) activity of fractions obtained by countercurrent distribution of *S. drummondii* butanol solubles.[a]

CCD fraction and recovered weight (g)	KB cytotoxicity ED_{50}, mcg/ml	Maximum PS activity T/C (dose, mg/kg)
1. 4.5	15.0	155 (80)
2. 3.5	2.5	184 (22)
3. 1.7	0.77	178 (10)
4. 1.3	0.40	170 (5)
5. 2.3	0.48	165 (5)
6. 2.5	1.2	152 (5)
7. 3.8	26.0	168 (10)
8. 3.2	2.4	168 (22)
9. 3.3	29.0	163 (180)
10. 0.2	24.0	142 (35)

[a]Solvent system was prepared by mixing water, ethyl acetate, and methanol in a 2:2:1 ratio.

Sugars and other hydrophilic compounds were prominent in ccd fractions 1 and 2, whereas fractions 7–10 were rich in lipophilic materials. Fractions 3–6 were highly cytotoxic (KB cell culture) and demonstrated *in vivo* PS activity at dose levels down to 0.5 mg/kg. These intermediate fractions proved to be rich in polyphenols including catechin, gallocatechin, and afzelechin. The scatter of high T/C values in all 10 ccd fractions indicates that several compounds of widely different polarity contribute to the observed PS activity. Scale-up of the ccd procedure gave comparable results for the remaining butanol solubles, and fractions 4 (455 g), 5 (453 g), and 6 (513 g) were selected for further study.

Removal of polyphenolic material was achieved by partition of ccd fractions between ethyl acetate and 5% aqueous sodium carbonate. Cytotoxic materials concentrated in the ethyl acetate layer. Further concentration of KB activity was achieved by chromatography on silica, chromatography on Sephadex G-10, and, finally, by hplc in two stages on reversed-phase columns. After 280-fold concentration, from ccd fractions 4–6 (1.4 kg) through chromatography on Sephadex (5.2 g), the cytotoxicity and PS activity of the most active fraction had not increased significantly. This observation suggests that the active principles of

S. drummondii are relatively labile and that extensive degradation of the active components occurred during these isolation steps.

Sesbanine (**1**) was isolated, 1.1 x 10^{-5}% yield, as one of a series of overlapping peaks from a C_{18} bonded column with water-methanol (9:1) as the eluting solvent. Crude **1** was cytotoxic, ED_{50}=4.7 x 10^{-2} mcg/ml. However, recrystallization from methanol gave **1**, mp 240–243°, which was only marginally cytotoxic and was inactive against PS *in vivo* at levels up to 1.4 mg/kg. The structure of **1** was revealed in an earlier x-ray crystallographic study (3) and has been confirmed by total synthesis (4, 5).

Drummondol (**2**) was isolated as a colorless oil, 3.2 x 10^{-5}% yield, with retention time approximately ¾ that of **1** on a C_{18} column under similar chromatographic conditions. Compound **2** was inactive against KB; ED_{50}=5.4 x 10^{1} mcg/ml. The structure of **2** was determined by detailed analysis of the ^{1}H and ^{13}C spectral data (table 2). Acylation of **2** with *p*-bromobenzoyl chloride gave **2a**, which

TABLE 2. ^{1}H and ^{13}C nmr assignments for drummondol (**2**).[a]

^{1}H assignment	Chemical shift[b]	^{13}C assignment	Chemical shift[b]
C–1 methyl	1.00 (s)	C–1	47.7 (s)
C–2 HA	2.60 (dd); J=18,2	C–1 methyl	15.4 (q)
HB	2.35 (d); J=18	C–2	52.6 (t)[c]
C–4 CH_2	2.61 (s)	C–3	206.9 (s)
C–5 methyl	1.20 (s)	C–4	53.1 (t)[c]
C–7 HA	3.88 (dd); J=8,2	C–5	81.9 (s)
HB	3.72 (d); J=8	C–5 methyl	18.9 (q)
C–1' vinyl H	5.86 (q); J=15,1	C–7	77.3 (t)
C–2' vinyl H	6.20 (q); J=15,6	C–8	85.7 (s)
C–3' H	4.45 (m); J=6,1	C–1'	124.1 (d)[d]
C–3' methyl	1.33 (d); J=6	C–2'	140.0 (d)[d]
		C–3'	68.0 (d)
		C–3' methyl	24.2 (q)

[a]Our numbering of **2** is that of a 6-oxabicyclo[3,2,1]-octane and differs from that of previous work (6, 8) which attempted to demonstrate the close relationship of **3** and **5**.

[b]Chemical shifts in ppm (δ) and coupling constants (J) as Hz (cps). Extensive decoupling was used to verify assignments.

[c]Assignments may be reversed.

[d]Assignments may be reversed.

exhibited a downfield shift of the C–3' proton signal from δ4.45 to δ5.58. The ^{1}H spectrum of **2** clearly shows that there is long range coupling (2 Hz) between the downfield C–7 oxymethylene signal (δ3.88) and the C–2 methylene signal centered at δ2.60. Other appropriate signals compare well with the ^{1}H spectrum of the methyl ester of phaseic acid (**3**) (6). The ms of **2** and **2a** were similar in that the highest ion observed was m/e 222 for both. This corresponded to M^{+}-H_2O for **2** and M^{+}-$C_7H_5O_2Br$ for **2a**.

It seems probable that **2** arises from **3** by oxidative removal of two carbons in a manner similar to that proposed for the formation of vomifoliol (**4**) from abscisic acid (**5**) (7). Alternatively, **2** could arise from **4** by an oxidative pathway similar to that demonstrated for the conversion of **5** to **3** (8).

We are continuing our investigations of fractions derived from *S. drummondii* seed extracts in an effort to establish the true antileukemic principles.

EXPERIMENTAL[2]

PLANT MATERIAL.—Seed of *Sesbania drummondii* (Rydb.) Cory was harvested from the wild in Texas during November 1975 (PR-44884). The collection was arranged and authenticated by Dr. Robert E. Perdue of the Medicinal Plant Resources Laboratory, USDA, Beltsville, Maryland.

EXTRACTION AND FRACTIONATION.—Seed material (454 kg) was ground in a Fitz mill to a particle size of 0.5–2.0 mm in diameter immediately prior to extraction. The ground material was placed in a 2270 liter tank containing 908 liters of 95% ethanol, heated to 50°, and stirred overnight. The ethanol extract was removed and the seed material was similarly extracted four more times, each with 450 liters of 95% ethanol. Ethanol extracts were combined and concentrated in a vacuum evaporator to a light syrup estimated to contain 25 kg of alcohol solubles. The concentrate was diluted with sufficient water to give 227 liters of an ethanol-water (1:3) solution, which was defatted by partitioning three times with a total of 400 liters of petroleum ether. Petroleum ether solubles amounted to 10.1 kg. Alcohol was then removed from the aqueous layer by concentrating to a volume of 45 liters; an equal volume of water was added, and the resulting solution was extracted once with 90 liters of 1-butanol and twice with 45 liters of 1-butanol. Butanol solubles were concentrated in an evaporator yielding 6.7 kg of a thick syrup.

Butanol soluble material, 6.6 kg, was then passed through a 10-carboy ccd in four batches. Each carboy contained 16 liters of a three-component biphasic solvent: water, ethyl acetate, and methanol in a 2:2:1 ratio, adjusted to equal phase volumes. Based on activity data of a preliminary small scale ccd (table 1) and similar data on the large scale ccd, fractions 4 (455 g), 5 (453 g), and 6 (513 g) were selected for further study. Each of the three ccd fractions was divided into two portions. To further concentrate activity of the six fractions thus obtained, each was dissolved in 9 liters of 5% Na_2CO_3 solution and extracted four times with 3 liters of ethyl acetate. In this manner, considerable polar and acidic (phenolic) material was removed and 238 g of ethyl acetate solubles remained. This material was divided into 15 portions ranging from 10–25 g; each portion was chromatographed on 250 g of silica in a 6 cm ID column. Each column was prepared in chloroform and eluted with a gradient consisting of 1.5 liters chloroform-ethyl acetate (1:1), 1.5 liters ethyl acetate, 1.5 liters ethyl acetate-methanol (1:1), and 0.75 liter of methanol. Forty 100-ml fractions collected from each run were combined into four, based on cytotoxicity the tlc similarity, and similar fractions from all 15 runs were combined (48 g). Tlc of this material on silica plates, developed with chloroform-methanol-water (40:10:1), gave many spots in the R_f 0.45–0.76 range.

The fractions of intermediate polarity, from chromatography on silica (48 g), were rechromatographed on a 3.5 cm ID column packed with Sephadex G-10 in 11 portions of 2–6 g each. The columns, prepared in water, were eluted in sequence with 1 liter of water, 500 ml of water-ethanol (1:1), and 500 ml of 95% ethanol. The first 1 liter fraction from each column was found to contain all of the cytotoxic material recovered and, when combined, it amounted to 5.2 g: $ED_{50} = 3.9 \times 10^{-1}$ μg/ml (KB) and T/C = 174 at 20 mg/kg (PS).

The active concentrate from chromatography on Sephadex was then subjected to reversed-phase liquid chromatography on a 2 m x 1 cm column packed with C_{18} Porasil B with water-methanol (4:1) as solvent at a flow rate of 9 ml/min. The most active fraction eluted with a retention time of 22–23 min and was resolved into a series of 16 peaks by further hplc on a C_{18} μ Bondapak column (30 cm x 7.9 mm) with the same solvent and a flow rate of 2 ml/min. Drummondol (**2**) was obtained as a sharp peak with retention time of 17.5 min, and sesbanine (**1**) was a major component of the most active fraction having a broad peak with retention time of 20–25 min. Further chromatography on the same column with water-methanol (9:1) gave crude **1**, retention time 65–73 min, $ED_{50} = 4.7 \times 10^{-2}$ μg/ml.

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[2]Melting points were determined on a Fisher-Johns block and are uncorrected. Optical rotations were determined with a Cary Model 60 recording spectropolarimeter and ir analyses were done on 1% solutions in $CHCl_3$ with a Perkin-Elmer model 700 instrument. Low-resolution mass spectra were obtained on a DuPont (CEC) 21-492-1 spectrometer, and high resolution data with a Kratos MS-30 instrument. ^1H nmr spectra were measured with a Varian XL-100 spectrometer and ^{13}C spectra were measured on a Bruker WH-90 Fourier transform spectrometer in $CDCl_3$ solutions unless stated otherwise. Hplc was carried out with a Waters model 6000 instrument equipped with a refractive index detector. Tlc analyses were carried out on plates spread with 0.25-mm layers of silica gel G (E. Merck, Darmstadt); components were visualized under uv or by charring with sulfuric acid-dichromate solution. Fractions and compounds were tested against P-388 lymphocytic leukemia in the mouse and Eagles 9KB nasopharyngeal carcinoma cell culture according to established protocols (9) under auspices of the National Cancer Institute. In the P-388 system an effective response is obtained when the median survival time of treated animals over control animals (% T/C) is ≥130%. In the KB cell culture system, results are expressed as that dose which inhibits growth to 50% of control growth (ED_{50}). An ED_{50} of 4 μg/ml is considered effective for pure compounds and 20 μg/ml for crude plant fractions.

SESBANINE (**1**).—Crude **1**, as obtained by hplc (50 mg) was recrystallized from methanol, mp 240–243°; $[\alpha]^{23}D$+14.6° (c 0.56, MeOH); ir 3510, 3490, 1710, 1690, 1600 cm^{-1} (KBr); ^1H nmr ($CDCl_3$-CD_3OD); δ9.26 (d, 1 Hz, H-8), 8.88 (dd, 6 and 1 Hz, H-6), 7.94 (d, 6 Hz, H-5); the H-10 proton appeared as a multiplet at 4.74 and remaining protons appeared in poorly resolved upfield multiplets; ^{13}C nmr (DMSO-d6); δ177.2 (C-1), 163.8 (C-3), 52.1 (C-4), 155.8 (C-4a), 121.7 (C-5), 148.3 (C-6), 153.9 (C-8), 119.6 (C-8a),[3] 48.5 (C-9), 72.7 (C-10), 36.1 (C-11), 42.4 (C-12): ^{13}C nmr (pyridine-d_5); δ178.5 (s, C-1), 165.0 (s, C-3), 53.2 (s, C-4), 156.6 (s, C-4a), 154.5 (d, C-8), 49.7 (t, C-9), 73.9 (d, C-10), 37.8 (t, C-11), 43.9 (t, C-12); ms, *m/e* (relative intensity): 232 (12), 214 (6), 204 (3), 199 (4), 175 (100), 162 (10), 157 (13), 129 (11). CI ms, found: M^++1, $C_{12}H_{13}N_2O_3$, *m/e* 233.0907; $C_{12}H_{13}N_2O_3$ requires 233.0888.

DRUMMONDOL (**2**).—Compound **2** was isolated as a colorless oil, 145 mg; $[\alpha]^{23}D = -21°$ (c 1.03, MeOH); ir ($CHCl_3$): 3700, 3500, 1725 cm^{-1}; ^1H and ^{13}C nmr (see table 2); ms, *m/e* (relative intensity): 222 (3), 204 (8), 164 (7), 136 (12), 125 (17), 123 (12), 99 (34), 82 (45), 71 (33), 69 (25), 55 (52), 43 (100).

DRUMMONDOL *p*-BROMOBENZOATE (**2a**).—A 36 mg portion of **2** was dissolved in pyridine (3 ml), 90 mg of p-bromobenzoyl chloride was added, and the resulting solution was allowed to stand overnight at 25°. Pyridine was removed (N_2), and the residue was partitioned between chloroform and water. Preparative tlc of the chloroform soluble material (silica, chloroform-methanol 97.5:2.5) then gave **2a** (20 mg) as an amorphous solid; ir ($CHCl_3$): 3630, 1720, 1600 cm^{-1}; ^1H nmr ($CDCl_3$): δ0.94 (s, 3H), 1.17 (s, 3H), 1.48 (d, 6Hz, 3H), 2.32 (d, 18Hz, 1H), 2.54 (s, 2H), 2.57 (dd, 18 and 2Hz, 1H), 3.68 (d, 8Hz, 1H), 3.86 (dd, 8 and 2Hz, 1H), 5.58 (m, 6Hz, 1H), 5.92 (d, 15.5Hz, 1H), 6.19 (q, 15.5 and 6Hz, 1H), 7.4–7.9 (m, 4H); ms, *m/e* (relative intensity): 222 (21), 204 (15), 185 (40), 183 (42), 164 (21), 136 (40), 125 (66), 123 (82), 82 (100). Found: M^+-$C_7H_5O_2Br$, *m/e* 222.1266; $C_{13}H_{18}O_3$ required 222.1255.

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[3]This signal was erroneously quoted (3) as overlapping with the C-5 signal (δ121.7). Assignments for C-5 and C-8a may be reversed.