Linear Furocoumarins (Psoralens) from the Seed of Texas *Ammi majus* L. (Bishop's Weed)

G. Wayne Ivie

Twelve linear furocoumarins (psoralens) were isolated from the ripened seed of a Texas collection of the photosensitizing weed *Ammi majus* L. Four of the compounds, xanthotoxin, bergapten, isopimpinellin, and isoimperatorin, have already been reported from this plant, and six of the eight other psoralens isolated are known plant products but have not been previously obtained from *A. majus*. Two of the *A. majus* psoralens, 8-[2-(3-methylbutyroxy)-3-hydroxy-3-methylbutoxy]psoralen and 5-[2-(acetoxy)-3-hydroxy-3-methylbutoxy]psoralen, have apparently not been previously isolated from any plant source. Six other synthetic psoralens were not observed in the seed extracts. Most of the major *A. majus* psoralens are potent photosensitizers, based on bioassays against chick skin, and the presence of these compounds in the seed and other parts of *A. majus* almost certainly accounts for the high photosensitizing activity of this plant toward cattle and sheep.

The Umbelliferous plant *Ammi majus* L. (Bishop's weed) has been used medicinally in the Middle East for centuries in the treatment of leukoderma (skin depigmentation, vitiligo) (Pathak et al., 1974). Not until the late 1940's, however, was it established that the photosensitizing and pigment stimulating agents in this plant were a group of compounds known as linear furocoumarins or psoralens. Egyptian workers isolated several substituted psoralens from *A. majus* and determined that the purified compounds and light were effective in treating leukoderma (Fahmy and Abu-Shady, 1948). *Ammi majus* and other photosensitizing plants are cultivated even today in some parts of the world as a source of these medicinally important compounds. Some psoralens are also synthesized for medicinal use in treating leukoderma and, more recently, psoriasis (Van Scott, 1976). A recent, extensive review deals with the biochemical basis for light-induced psoralen reactions with skin (Scott et al., 1976).

Ammi majus also induces phototoxic responses in livestock and poultry. Young geese and ducks that have eaten as little as (0.5 g of Israeli *A. majus* seed) $bird^{-1}$ day^{-1} experienced a severe photosensitization syndrome after exposure to sunlight. The symptoms included blistering and subsequent deformation of the beak, feet, wings, and ocular areas (Eilat et al., 1974; Egyed et al., 1974a, 1975a,b). Dairy cattle were also affected after consumption of *A. majus* contaminated feed and subsequent exposure to sunlight (Egyed et al., 1974b).

Although *A. majus* is primarily an Old World species, it grows in the coastal regions of the southern United States and in some areas as far as 200 km or more inland. In Texas, periodic outbreaks of photosensitization affecting thousands of cattle have been observed for many years (Dollahite et al., 1978). The distribution of these outbreaks coincides with the distribution of *A. majus*, and recent studies in our laboratory have shown that experimental feeding of the Texas *A. majus* to cattle or sheep, followed by exposure to sunlight, induces a photosensitization syndrome very similar to that seen in the field (Dollahite et al., 1978; Witzel et al., 1978). The photosensitization involves severe blistering and peeling in light-skinned animals and the development of cloudy corneas that can apparently lead to blindness (Witzel et al., 1978).

The studies reported here were undertaken to evaluate the furocoumarin chemistry of Texas *A. majus*. Although considerable efforts have been directed toward the isolation and structure elucidation of *A. majus* furocoumarins by Egyptian and, to a lesser extent, European workers, such work apparently has not been undertaken on North American *A. majus*. The *A. majus* psoralens, as well as several additional synthetic compounds, were also tested for photosensitizing activity against the skin of newly hatched chicks.

Veterinary Toxicology and Entomology Research Laboratory, Agricultural Research, Science and Education Administration, U.S. Department of Agriculture, College Station, Texas 77840.

MATERIALS AND METHODS

Analytical Procedures. Mass spectra were recorded by direct insertion probe analysis on a Varian/MAT CH-7 magnetic scan spectrometer. Nuclear magnetic resonance (NMR) spectra were recorded mostly using a JEOL Model JNM-MH-100 spectrometer, but some were done in the Varian NMR Applications Laboratory, Palo Alto, Calif., with a 300-MHz superconducting Fourier transform (FT) NMR system. Samples were run in deuterated chloroform or acetone, using tetramethylsilane (Me_4Si) as an internal reference. Chemical shifts are reported in parts per million downfield from Me_4Si. Melting points (uncorrected) were recorded in open capillary tubes with a Tottoli-type melting point apparatus. Optical rotation studies were done in chloroform at the D line of sodium with a Cary 60 Spectropolarimeter.

Extraction of Seed. Mature seed heads of *A. majus* were collected during June, 1976, in Wharton County, Texas; plants were identified by J. W. Dollahite of this laboratory. The ripened seeds, which dislodged easily as the plants dried, were collected and held frozen until analysis.

Whole seed (100 g) was extracted with 250 mL of chloroform by blending thoroughly with a Willems Polytron Homogenizer. The slurry was filtered under vacuum through a medium porosity fritted glass funnel, and the residue was extracted three more times with chloroform. The combined chloroform extracts were dried over sodium sulfate, and the chloroform was removed by vacuum distillation. The residue was partitioned between acetonitrile and hexane (100 mL each) to remove lipids, and the hexane phase was discarded. The acetonitrile was adjusted to 100 mL and this phase, containing 1 g equiv of *A. majus* seed extract/mL, was subjected to thin-layer chromatographic (TLC) analysis.

Chromatography. TLC was used to resolve and isolate the psoralen constituents from the seed extracts. Precoated silica gel plates (20 × 20 cm, Brinkman Silplate F-22, with fluorescent indicator, 0.25, 0.5, or 2.0 mm gel thickness) were used in all separations. With these plates, the substituted psoralens present were easily detected as yellow or golden bands by viewing the developed plates

Table I. TLC R_f Values for *Ammi majus* Psoralens and Related Compounds

compound[a]	R_f in indicated solvent system[b,c]					
	1[d]	2	3	4	5	6
1	0.42	0.45	0.54	0.17	0.47	0.55
2	0.53	0.47	0.58	0.21	0.50	0.58
3	0.36	0.47	0.55	0.16	0.48	0.57
4	0.57	0.60	0.65	0.28	0.63	0.67
5	0.27	0.45	0.55	0.10	0.54	0.57
6	0.17	0.40	0.51	0.07	0.46	0.53
7	0.01	0.05	0.06	0.01	0.29	0.08
8	0.14	0.35	0.44	0.03	0.54	0.49
9	0.09	0.29	0.38	0.02	0.46	0.43
10	0.05	0.27	0.32	0.01	0.50	0.37
11	0.02	0.22	0.26	0.01	0.41	0.32
12	0.03	0.16	0.19	0.01	0.39	0.23
13	0.05	0.20	0.28	0.03	0.43	0.34
14	0.07	0.22	0.32	0.02	0.36	0.36
15	0.50	0.52	0.60	0.22	0.54	0.62
16	0.00	0.06	0.06	0.00	0.25	0.08
17	0.10	0.32	0.43	0.04	0.45	0.45
18	0.01	0.12	0.15	0.01	0.30	0.19

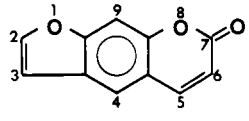
[a] For chemical names of compounds see Table III. [b] Brinkman Silplate F-22, 0.25 mm gel thickness. [c] Solvent systems as follows: 1 (methylene chloride); 2 (chloroform-ethyl acetate, 2:1); 3 (methylene chloride-ether, 2:1); 4 (benzene-ether, 20:1); 5 (hexane-ethyl acetate-methanol, 5:5:1); and 6 (methylene chloride-ether, 1:1). [d] Plates developed three times.

under long-wave ultraviolet (UV) light. Psoralens were isolated by applying the extract as bands to TLC plates, developing in appropriate solvent systems, locating the compounds under UV light, and then scraping and eluting with chloroform or acetone. Compounds were crystallized directly from appropriate solvents (usually ether or ether–hexane), or if necessary, the compounds were subjected to further cleanup by TLC. The solvent systems used and the numbers used to designate them throughout this report are as follows: 1, methylene chloride; 2, chloroform–ethyl acetate (2:1); 3, methylene chloride–ether (2:1); 4, benzene–ether (20:1); 5, hexane–ethyl acetate–methanol (5:5:1); and 6, methylene chloride–ether (1:1). TLC R_f values for each of the psoralens studied are indicated in Table I.

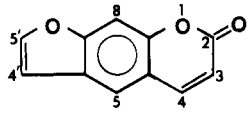
Synthesis. In some cases, chemical modifications of the *A. majus* psoralens were made to verify their structures. Other psoralens were synthesized to confirm the structural assignments of *A. majus* constituents and to provide TLC standards for determining their possible presence in the plant extracts. All structures were verified by mass spectral and NMR analysis and by comparison with previously published data on the compounds where possible. One of the compounds isolated from the *A. majus* extracts, xanthotoxin (8-methoxypsoralen), was also obtained in gram quantities commercially (Biochemical Laboratories, Redondo Beach, Calif.) and was used in synthetic studies involving this compound because larger quantities were available.

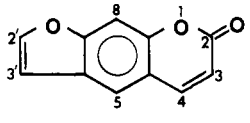
Phototoxicity. Compounds were tested for photosensitizing activity against the skin of 1- to 3-day-old chicks (mixed sex, White Leghorn, Kazmeier Hatchery, Bryan, Tex.). The down was removed from the skin over the skull cap by application of a commercial depilatory (Neet lotion, Whitewall Laboratories, New York, N.Y.), followed 1–3 min later by gentle rubbing with a soft cloth. The various furocoumarins [up to 100 μg in 10 μL of dimethyl sulfoxide (Me_2SO) solution] were injected subcutaneously under the bare skin, then the chicks were exposed for 24 h to overhead long-wave UV light (Sylvania blacklite, F 30T8-BL, two 90-cm fluorescent tubes spaced about 7 cm apart). [It is well known that long-wave UV is the primary radiation effecting psoralen photosensitization (Scott et al., 1976).] During the exposure, the chicks were held in a cardboard box 84 cm long × 25 cm wide × 25 cm deep, and the lights were rested lengthwise across the top of the box. Controls consisted of chicks that were injected with 10 μL of Me_2SO only and exposed to light. In tests to determine if the responses were light dependent, chicks were also injected with the highest level of photosensitizer tested and then exposed only to indirect room lighting and not to long-wave UV. At least three birds were tested at each photosensitizer concentration and for both light-exposed and nonexposed controls. After the exposure period, the birds were transferred to incubators, and the phototoxic effects were evaluated 6 days later as follows: (–) no response; (+) definite thickening of the skin; (++) skin blistered; (+++) severe phototoxicity characterized by darkened, dried, and hard skin.

Nomenclature. For the past decade, Chemical Abstracts (CA) has named psoralens as derivatives of 7*H*-furo[3,2-*g*][1]benzopyran-7-one, in which the two aromatic protons are numbered 4 and 9. Almost without exception,



however, investigators in this field continue to use either of two older systems of nomenclature in which the compounds are named as "psoralens", and the aromatic protons are numbered 5 and 8. In the current report, the





alkoxy psoralens isolated from *A. majus* or synthesized chemically are named as 5- or 8-substituted derivatives. Thus, xanthotoxin (8-methoxypsoralen in this report) is listed by CA as the 9-methoxy derivative of 7*H*-furo[3,2-*g*][1]benzopyran-7-one. The nomenclature of the alkoxy substituents in this paper is strictly according to that in CA.

RESULTS

Preliminary TLC studies with the extract of *A. majus* seed indicated that multiple development of 0.25-mm plates in methylene chloride (solvent system 1) gave good resolution of several components. On the basis of their appearance under long-wave UV light, each of the bands

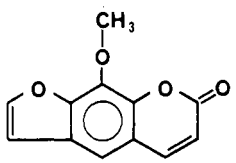
Table II. Yield, Melting Point, and Optical Rotation Data for Substituted Psoralens from *Ammi majus* Seed and for Related Synthetic Psoralens

	compound[a]	yield, %	mp, °C	literature mp, °C	specific rotation $[\alpha]^{25}D$[b]
1	(xanthotoxin)	0.36	144.5-145.0	148 (Schonberg and Sina, 1950)	*c*
2	(bergapten)	0.11	188.0-189.0	185-187 (Adityachaudhury et al., 1974)	*c*
3	(isopimpinellin)	0.23	147.0	148 (Talapatra et al, 1973)	*c*
4	(isoimperatorin)	<0.01	97.0	108-109 (Atkinson et al., 1974)	*c*
5	(oxypeucedanin)	0.30	102.0	102-103 (Atkinson et al., 1974)	+11.8°
6	(heraclenin)	0.07	105.0	107-108 (Adityachaudhury et al., 1974)	-7.5°
7	(oxypeucedanin hydrate)	0.04	126.5-128.5	125-128 (Atkinson et al., 1974)	+9.9°
8	(saxalin)	<0.01	142.5-144.0	159-161 (Avramenko and Nikonov, 1971)	*d*
9	(pabulenol)	<0.01	121.0-122.0	120-121 (Gonzalez et al., 1973)	*d*
10		0.10	109.0-110.0		-26.4°
11		0.01	oil		*d*
12		0.01	141.0-142.0		*d*
13	(xanthotoxol)	synthetic	236.0-240.0	246 (Schonberg and Sina, 1950)	*c*
14	(bergaptol)	synthetic	268.0-270.0	270 (Stanley and Vannier, 1957)	*c*
15	(imperatorin)	synthetic	96.0-97.0	98-99 (Siddiqui and Sen, 1972)	*c*
16	(heraclenol)	synthetic	127.0-128.0	114-115 (Adityachaudhury et al., 1974)	*e*
17		synthetic	103.0-104.0	103-104 (Gonzalez et al., 1974)	*e*
18		synthetic	oil		*e*

[a] For chemical names of compounds, see Table III. [b] Optical rotation studies done in chloroform. [c] No asymmetric centers. [d] Insufficient quantities available for measurement. [e] Racemic synthetic mixture.

was suspected to contain psoralens. To obtain sufficient quantities of these products for studies of their chemical nature, the extract was applied to preparative TLC plates (2.0 mm) that were subsequently developed three times in solvent system 1. The components as eluted from the appropriate gel regions were analyzed as discussed below. The yields of the psoralens obtained from the seed extracts are shown in Table II, as are melting point and optical rotation data.

Xanthotoxin (8-Methoxypsoralen, 1). This compound showed the same TLC behavior as an authentic sample of xanthotoxin. It was crystallized from ether, and the purified 1 exhibited NMR and mass spectral behavior



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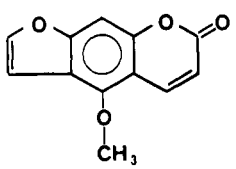
identical with that of authentic 1 (Tables III and IV).

Xanthotoxin is one of the most widely distributed plant psoralens and was the first psoralen to be isolated from *A. majus* (Fahmy et al., 1947).

Bergapten (5-Methoxypsoralen, 2). This product was crystallized from ether, and mass spectral analysis indicated it to be isomeric with 1 (Table IV). NMR analysis (Table III) confirmed that the product was bergapten (2),



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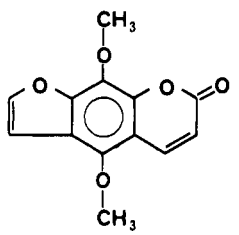
a psoralen derivative previously isolated from numerous plant sources, including *A. majus* (Fahmy and Abu-Shady, 1948).

The NMR spectra of 1 and 2 show distinct differences between these two isomers (Table III), and subsequent studies with other alkoxy psoralens from the *A. majus* seed indicated that, regardless of the nature of the alkoxy substituent, 5- or 8-substitution was clearly indicated by NMR. In the 8-alkoxy series, the H_4 and $H_{5'}$ signals overlap, but in the 5-substituted compounds, H_4 is shifted downfield and $H_{5'}$ upfield. The aromatic proton H_5 in the 8-alkoxy psoralens always appeared between δ 7.3–7.4, whereas H_8 in the 5-alkoxy series was at δ 7.0–7.2 (Table III).

Isopimpinellin (5,8-Dimethoxypsoralen, 3). This component was crystallized as yellow needles from ether and identified by spectral analysis as isopimpinellin (3).



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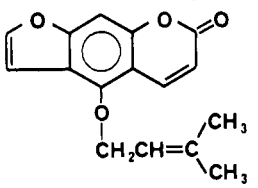
NMR of the product (Table III) clearly indicated 5,8-disubstitution due to the absence of both H_5 and H_8 signals. As previously reported, the two *O*-methyl groups of 3 do not give separate signals but rather a six-proton singlet at δ 4.12 (Steck and Bailey, 1969). The mass spectrum of 3 (Table IV) was consistent with the assigned structure. Isopimpinellin is a widely distributed plant psoralen and has previously been reported as a constitutent of *A. majus* (Abdel-Hay et al., 1966).

Isoimperatorin (5-[(3-Methyl-2-butenyl)oxy]psoralen, 4). The component of highest TLC R_f in solvent system 1 (Table I) did not satisfactorily resolve from 2 during preparative TLC. This did not affect the purification of 2, but further cleanup was required before the very small amounts of 4 could be crystallized. Purification



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of 4 was effected by TLC in solvent system 4 (developed twice). The purified compound was subsequently crys-

Table III. NMR Data for *Ammi majus* Psoralens and Related Compounds

compd	chemical name	protons[a,b]						
		H_3	H_4	H_5	H_8	$H_{4'}$	$H_{5'}$	others
1	8-methoxypsoralen	6.32 d	7.72 d	7.32 s		6.80 d	7.64 d	4.26 s (OCH_3)
2	5-methoxypsoralen	6.24 d	8.12 d		7.10 s	7.00 d	7.56 d	4.24 s (OCH_3)
3	5,8-dimethoxypsoralen	6.25 d	8.08 d			6.96 d	7.60 d	4.12 s (2 OCH_3)
4	5-[(3-methyl-2-butenyl)oxy]-psoralen	6.27 d	8.15 d		7.17 s	7.05 d	7.59 d	5.59 t (C=CH), 4.97 d (OCH_2), 1.76 s (CH_3), 1.65 s (CH_3)
5	5-[(3,3-dimethyloxiranyl)methoxy]-psoralen	6.29 d	8.19 d		7.18 s	6.95 d	7.60 d	4.52 m (OCH_2), 3.22 t (CH), 1.41 s (CH_3), 1.32 s (CH_3)
6	8-[(3,3-dimethyloxiranyl)methoxy]-psoralen	6.36 d	7.78 d	7.40 s		6.84 d	7.70 d	4.60 d (OCH_2), 3.32 t (CH), 1.34 s (CH_3), 1.28 s (CH_3)
7	5-(2,3-dihydroxy-3-methylbutoxy)-psoralen	6.19 d	8.17 d		7.09 s	6.97 d	7.59 d	4.43 m (OCH_2), 3.87 m (CHOH), 3.09 br (OH), 2.36 br (OH), 1.33 s (CH_3), 1.29 s (CH_3)
8	5-(3-chloro-2-hydroxy-3-methylbutoxy)psoralen	6.24 d	8.16 d		7.12 s	6.98 d	7.60 d	4.60 m (OCH_2), 4.10 m (C*H*OH), 2.80 d (OH), 1.70 s (2 CH_3)
9	5-[(2-hydroxy-3-methyl-3-butenyl)-oxy]psoralen	6.29 d	8.17 d		7.17 s	6.96 d	7.60 d	5.19-5.06 s ($C=CH_2$), 4.55 m (C*H*OH), 4.42 m (OCH_2), 1.83 s (CH_3)
10	5-[2-(3-methylbutyloxy)-3-hydroxy-3-methylbutoxy]psoralen	6.22 d	8.06 d		7.10 s	6.96 d	7.56 d	5.32 m (C*H*OR), 4.68 m (OCH_2), 2.22 m ($COCH_2CH$), 1.75 br (OH), 1.36 s (CH_3), 1.32 s (CH_3), 0.95 d [$CH(CH_3)_2$]
11	8-[2-(3-methylbutyloxy)-3-hydroxy-3-methylbutoxy]psoralen	6.30 d	7.72 d	7.34 s		6.78 d	7.66 d	5.22 m (C*H*OR), 4.70 m (OCH_2), 2.25 m ($COCH_2CH$), 1.66 br (OH), 1.36 s (CH_3), 1.32 s (CH_3), 0.95 d [$CH(CH_3)_2$]
12	5-[2-(acetoxy)-3-hydroxy-3-methylbutoxy]psoralen	6.28 d	8.04 d		7.12 s	6.96 d	7.56 d	5.34 m (CHOR), 4.71 m (OCH_2), 2.08 s ($COCH_3$), 1.32 s (CH_3), 1.28 s (CH_3)
13[a]	8-hydroxypsoralen	6.48 d	8.26 d	7.64 s		7.16 d	8.14 d	3.10 br (OH)
14[a]	5-hydroxypsoralen	6.12 d	8.41 d		7.06 s	7.30 d	7.82 d	
15	8-[(3-methyl-2-butenyl)oxy]-psoralen	6.36 d	7.70 d	7.32 s		6.80 d	7.64 d	5.68 t (C=CH), 5.08 d (OCH_2), 1.68 s (2 CH_3)
16	8-(2,3-dihydroxy-3-methylbutoxy)-psoralen	6.29 d	7.73 d	7.33 s		6.77 d	7.67 d	4.65-4.29 m (OCH_2), 3.75 m (C*H*OH), 3.64 br (OH), 2.80 br (OH), 1.36 s (CH_3), 1.32 s (CH_3)
17	8-(3-chloro-2-hydroxy-3-methyl-butoxy)psoralen	6.33 d	7.79 d	7.43 s		6.81 d	7.71 d	4.79-4.35 m (OCH_2), 4.01 m (C*H*OH), 3.26 d (OH), 1.76 s (2 CH_3)
18	8-[2-(acetoxy)-3-hydroxy-3-methylbutoxy]psoralen	6.29 d	7.77 d	7.35 s		6.79 d	7.67 d	5.19 m (CHOR), 4.87-4.45 m (OCH_2), 2.24 s ($COCH_3$), 1.40 s (CH_3), 1.28 s (CH_3)

[a] Compounds 13 and 14 were run in deuterioacetone, all others in deuteriochloroform. [b] Chemical shifts in parts per million downfield from tetramethylsilane.

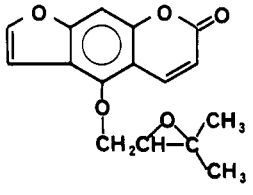
Table IV. Mass Spectral Data for *Ammi majus* Psoralens and Related Compounds

compd[a]	m/e: parent ion	base peak	other ions[b]
1	216	216	201 (P – CH_3), 188 (P – CO) 173 (P – CO,CH_3)
2	216	216	201, 188, 173
3	246	231 (P – CH_3)	216 (P – 2CH_3), 203 (P – CO,CH_3), 188 (P – CO,2CH_3)
4	270	202 (PsorOH)	215 (PsorOCH_2), 201 (PsorO), 174 (PsorOH – CO)
5	286	202	215, 201, 187 (PsorOCH_2 – CO), 174
6	286	202	215, 201, 187, 174
7	304	202	289 (P – CH_3), 245 (PsorOCH_2CHOH), 215, 201, 187, 174
8	322	202	286 (P – Cl, H), 245, 215, 201, 187, 174
9	286	202	215, 201, 187, 174
10	388	85 [$COCH_2CH(CH_3)_2$]	289 (PsorOCH_2CHOHCOHCH_3), 245, 215, 202, 201, 187, 174
11	388	85	289, 245, 215, 202, 201, 187, 174
12	346	202	331 (P – CH_3), 289, 245, 215, 202, 201, 174
13	202	174	201, 146 (P – CO_2, CH), 145 (P – CO_2CH_2)
14	202	174	201, 146, 145
15	270	202	215, 201, 174
16	304	202	289, 245, 215, 201, 174
17	322	202	245, 215, 201, 174
18	346	202	289, 245, 215, 201, 174

[a] For chemical names of compounds, see Table III. [b] Abbreviations as follows: P = parent ion, Psor = psoralen nucleus.

tallized from ether–hexane in <0.01% yield. NMR showed it to be a 5-substituted psoralen and, upon analysis of the NMR and mass spectral data (Tables III and IV), it was concluded that 4 contained a 3-methyl-2-butenyloxy side chain. The compound is thus the same as isoimperatorin, a psoralen previously isolated from several plant sources, including *A. majus* (Abu-Mustafa et al., 1968).

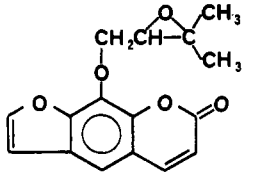
(+)-Oxypeucedanin (5-[3,3-Dimethyloxiranyl)-methoxy]psoralen, 5). This compound was crystallized from ether–hexane and, based upon its NMR spectrum (Table III), it was clearly a 5-substituted psoralen that was closely related to 4. The mass spectrum (Table IV) indicated that one atom of oxygen had been added to 4, and thus it seemed likely that the compound might be the epoxide of 4. NMR analysis confirmed this to be the case; NMR data for 5 (Table III) were essentially identical with



5

those previously reported for oxypeucedanin (Steck and Bailey, 1969). Oxypeucedanin is known to occur in many plant species, but it has not previously been reported from *A. majus*.

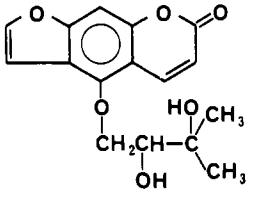
(–)-Heraclenin (8-[(3,3-Dimethyloxiranyl)methoxy]psoralen, 6). Crystallization of this compound from ether–hexane gave a product whose NMR and mass spectra (Tables III and IV) clearly showed it to be the 8-substituted isomer of 5, heraclenin. Further proof of structure of 6 as heraclenin was provided by synthesis of



6

the compound from xanthotoxol (8-hydroxypsoralen, vide infra). The synthetic 6 was identical (TLC, NMR, mass spectra) with 6 isolated from the *A. majus* seed extract.

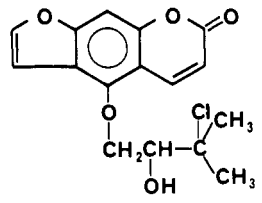
(+)-Oxypeucedanin hydrate (5-(2,3-Dihydroxy-3-methylbutoxy)psoralen, 7). TLC of the *A. majus* seed extract in solvent system 1 (Table I) did not resolve several components of low R_f. However, six suspected psoralens were seen in this fraction after TLC in solvent system 3 and visualization under long-wave UV light. The most polar component of these compounds was crystallized from ether–hexane, and its NMR spectrum (Table III) indicated it to be a 5-substituted psoralen with similarities to 5. The compound contained two apparent hydroxyl groups at δ 2.36 and 3.09 that were confirmed by deuterium exchange. On the basis of the NMR data, 7 was concluded to be



7

oxypeucedanin hydrate, generated by hydrolysis of the epoxide moiety of 5. Mass spectral data (Table IV) were consistent with the assigned structure. The structure of 7 was confirmed by hydrolysis of the epoxide 5 to 7 (vide infra).

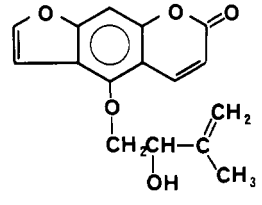
Saxalin [5-(3-Chloro-2-hydroxy-3-methylbutoxy)psoralen, 8]. This product was crystallized in very low yield from ether–hexane. Its mass spectrum (Table IV) showed it to contain a single chlorine atom, as evidenced by the 1-chlorine "cluster" molecular ion at *m/e* 322 (Cl = 35). Although <1 mg of the crystalline compound was obtained, it was successfully analyzed by FT-NMR (Table III). The pattern in the aromatic region indicated that 8 was a 5-substituted psoralen, and analysis of the side-chain protons strongly indicated that 8 was the chlorohydrin of 5. It was concluded that the chlorine was on the carbon with the two methyls due to the downfield shift of the methyl resonance (six-proton singlet at δ 1.70). The compound was thus assigned as saxalin, 5-(3-chloro-2-hydroxy-3-methylbutoxy)psoralen, a product previously reported from apparently only one other plant source, *Angelica saxatilis* (Avramenko and Nikonov, 1971). The structural assignment of 8 was confirmed by its synthesis from the epoxide precursor 5 (vide infra). Al-



8

though the melting point was the same for both the synthetic 8 and 8 from *A. majus*, it differs from the melting point for saxalin as reported by Avramenko and Nikonov (1971) (Table II). Sufficient quantities of 8 from *A. majus* were not available for studies of its optical activity.

Pabulenol (5-[(2-Hydroxy-3-methyl-3-butenyl)-oxy]psoralen, 9). This compound was obtained in very low yield by crystallization from ether–hexane. FT-NMR analysis of 9 showed it to be a 5-substituted psoralen

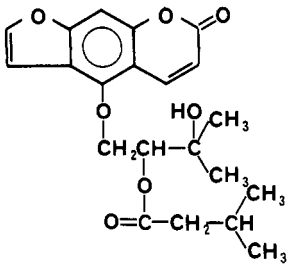


9

(Table III), and analysis of the side chain protons, in conjunction with the mass spectrum (Table IV), indicated that 9 was 5-[(2-hydroxy-3-methyl-3-butenyl)oxy]psoralen. The two vinyl protons gave a signal (multiplet) centered at δ 4.42, whereas the methine proton was at δ 4.55. The two additional signals at δ 5.06 and 5.19 were assigned to the two methylene protons. Integration of the spectrum indicated that the methyl signal corresponded to just three protons, which is consistent with the assigned structure. This compound has been isolated previously from several plant sources, and the spectral data obtained for 9 agreed with that previously published (Chatterjee et al., 1972; Gonzalez et al., 1973). Insufficient quantities of 9 were available for optical rotation studies.

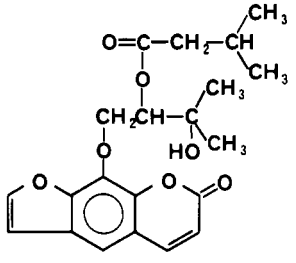
(−)-5-[2-(3-Methylbutyroxy)-3-hydroxy-3-methylbutoxy]psoralen (10). This product was crystallized from ether–hexane, and its mass spectrum (Table IV) showed the molecular ion at *m/e* 388, indicative of a rather large substituent on the psoralen nucleus. NMR of 10 showed it to be a 5-substituted psoralen (Table III), and, due to the complexity of the compound as indicated by NMR, initial attempts at its characterization were focused on chemical degradation.

Fifteen milligrams of 10 were dissolved in 10 mL of 50% methanolic potassium hydroxide (1.0 N). The solution was stirred for 10 min at room temperature, then was adjusted to pH 2.0, and the methanol was removed by vacuum distillation. The residual water phase was then extracted with ether, and the ether was concentrated to about 1 mL. Cooling effected crystallization, and the product obtained was identical in all respects (melting point, TLC, NMR, mass spectra) to the diol 7 already isolated from the *A. majus* seed extract. This finding, along with the mass spectral and NMR data, made it most likely that 10 was the isovaleric acid ester of 7. Accordingly, this ester was synthesized by reaction of 7 with isovaleryl chloride (vide infra). The synthetic compound proved to be identical (melting point, TLC, NMR, mass spectra) with 10 isolated from the plant. The product was therefore identified as 5-[2-(3-methylbutyroxy)-3-hydroxy-3-methylbutoxy]psoralen, a compound isolated previously from only one other plant source, *Seseli libanotis* (Bohlmann et al., 1968).



10

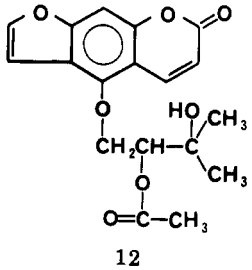
8-[2-(3-Methylbutyroxy)-3-hydroxy-3-methylbutoxy]psoralen (II). This compound was isolated in very low yield from the *A. majus* seed extract, and all efforts at crystallization were unsuccessful. Mass spectral and NMR analysis of 11 clearly showed it to be the 8-sub-



11

stituted isomer of 10 (Tables III and IV). In addition, synthesis of 11 from xanthotoxol through several intermediates (vide infra) provided further proof of structure. The synthetic compound and 11 isolated from the *A. majus* seed extract were identical (TLC, NMR, mass spectra). This report is apparently the first record of 11 as a natural product. Insufficient quantities of the compound were available to permit optical rotation studies.

5-[2-(Acetoxy)-3-hydroxy-3-methylbutoxy]psoralen (12). This compound was crystallized from ether–hexane and was identified as the acetyl ester of 7 on the basis of NMR and mass spectral analysis (Tables III and IV). Synthesis of 12 from 7 and acetyl chloride (vide infra)

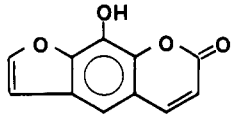


12

confirmed the structural assignment. The synthetic 12 was identical (melting point, TLC, NMR, mass spectra) to 12 isolated from *A. majus*. This compound has apparently not been previously reported from any plant source. Its optical activity was not determined because of the small quantities available.

The *A. majus* psoralens 10, 11, and 12 each have two esterifiable hydroxyls, but it was concluded that the secondary hydroxyl was the one esterified in each case due both to normally greater ease of reaction at this position and to the NMR data obtained. NMR of the esters 10, 11, and 12 showed downfield shifts of the CHOR and OCH_2 protons in relation to the unesterified diols 7 and 16. Also, the $C(OH)(CH_3)_2$ signals in the esters were essentially unchanged from the respective diol precursors, providing further evidence that the tertiary hydroxyls remained unreacted and that esterification was indeed at the secondary carbon.

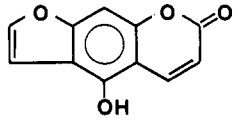
Synthetic Psoralens: Xanthotoxol (8-Hydroxypsoralen, 13). Demethylation of xanthotoxin (1) was effected using the procedure of Schonberg and Sina (1950). Into a 250-mL round-bottom flask were added 5.08 g of iodine and 0.49 g of magnesium metal. To this was added 60 mL of a 1:1 mixture of dry ether and dry benzene. The mixture was stirred for 30 min in an oil bath (room temperature), then 2.15 g of 1 was added, and the solvent was removed by gently heating the oil. After almost all of the solvent had volatilized, the flask was put under vacuum (<50 μmHg) and the bath temperature was raised to 160–170 °C. After 2 h, the flask was removed from the heat, and the residue was pulverized and then suspended in 0.1 N sulfuric acid (200 mL). This mixture was transferred to centrifuge bottles, extracted nine times with equal volumes of chloroform using Polytron homogenization, and then centrifuged to separate the phases. The chloroform extracts were combined, dried over sodium sulfate, then concentrated by vacuum distillation to 50–100 mL. Upon cooling, the product crystallized (1.80 g), and it was identified as xanthotoxol by NMR and mass spectral analysis (Tables III and IV). Reaction of **13** with diazo-



13

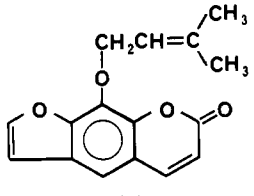
methane, followed by crystallization from ether, regenerated 1 (melting point, TLC, NMR, mass spectra).

Bergaptol (5-Hydroxypsoralen, 14). Bergaptol was prepared by demethylation of **2** using the procedure described above for synthesis of **13**, but on a much smaller scale (40 mg of **2**) due to the limited quantities of **2** available. The bergaptol obtained was purified on TLC (solvent system 3) and did not crystallize from chloroform but rather gave a tan solid (10 mg) upon evaporation of the solvent. The compound was identified as bergaptol on the basis of NMR and mass spectra (Tables III and IV) and by its conversion to **2** upon reaction with diazomethane (TLC, mass spectra).



14

Imperatorin (8-[(3-Methyl-2-butenyl)oxy]psoralen, 15). A mixture of 2.0 g of **13**, 5.0 g of potassium carbonate, 100 mL of acetone, and 5.0 mL of 1-bromo-3-methyl-2-butene was refluxed for 2 h. The mixture was then filtered to remove the carbonate. The acetone was removed by vacuum distillation, and then the sample was held under high vacuum (<50 μmHg) to remove all traces of the unreacted bromide. The reaction product was dissolved in 100 mL of ether, which was then concentrated to 10–15 mL. Upon cooling, 1.80 g of **15** crystallized, and an ad-

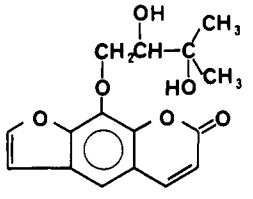


15

ditional 0.60 g of **15** was subsequently harvested from the mother liquor. NMR and mass spectral data (Tables III and IV), upon comparison with those of the 5-substituted isomer **4**, confirmed the product's structure.

Heraclenin (8-[(3,3-Dimethyloxiranyl)methoxy]psoralen, 6). A solution of 1.35 g of **15** in 30 mL of methylene chloride was stirred in a 100-mL round-bottom flask, and 2.0 g of *m*-chloroperoxybenzoic acid was added slowly to the mixture. After 30 min, the mixture was partitioned with an equal volume of 10% sodium sulfite to remove the excess peracid. The aqueous phase was back-extracted once with methylene chloride, and the combined organic fractions were dried over sodium sulfate, then the solvent was removed by vacuum distillation. The residue was crystallized from ether (two crops) to give a total of 1.17 g of **6**. The synthetic **6** was identical (melting point, TLC, NMR, mass spectra) to **6** isolated from the *A. majus* seed extracts.

Heraclenol [8-(2,3-Dihydroxy-3-methylbutoxy)psoralen, 16]. The epoxide **6** (0.72 g) was dissolved in 50 mL of 0.1 N sulfuric acid in 50% aqueous tetrahydrofuran. The mixture was stirred at room temperature for 2 h, then the THF was removed by vacuum distillation. The water phase was extracted nine times with ether, which was then dried over sodium sulfate and concentrated. The reaction product separated from ether–hexane first as an oil but was subsequently crystallized from ether to give a total of 0.66 g of a product that was identified as the diol **16** based

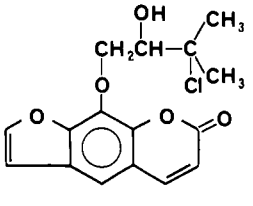


16

on NMR and mass spectral analysis in comparison with data from the 5-substituted isomer **7** (Tables III and IV).

Saxalin [5-(3-Chloro-2-hydroxy-3-methylbutoxy)psoralen, 8]. The epoxide **5** (15 mg) was dissolved in 4 mL of dry ether that had been saturated with HCl gas. The mixture was stirred in an ice bath for 5 min, then the excess acid was neutralized with sodium bicarbonate. The ether was removed with a pipet and the residue was washed twice with ether. Without further cleanup, the combined ether phases were concentrated and crystallization was effected by adding hexane. The crystalline product (12 mg) exhibited the same melting point, TLC behavior, and NMR and mass spectra as did **8** isolated from the plant extract.

8-(3-Chloro-2-hydroxy-3-methylbutoxy)psoralen (17). This chlorohydrin was synthesized from the epoxide **6** by the same procedure described above for the preparation of **8** from **5**. A total of 81 mg of the chlorohydrin **17** was obtained from 100 mg of **6**. The product was



17

identified on the basis of NMR and mass spectral data in comparison with that from the 5-substituted isomer **8** (Tables III and IV).

Isomeric 5- and 8-[2-(3-Methylbutyroxy)-3-hydroxy-3-methylbutoxy]psoralens (10 and 11). These

Table V. Phototoxicity of *Ammi majus* Psoralens and Related Compounds to the Skin of 1- to 3-Day-Old Chicks

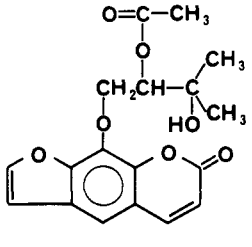
		phototoxic response at indicated treatment level (μg)[a,b]					
	compd[c,d]	0[e]	3.3	10	33	100	100 (dark)[f]
1	(xanthotoxin)	−	+	+++	+++	+++	−
2	(bergapten)	−	+	++	+++	+++	−
3	(isopimpinellin)	−	−	−	+	++	−
5	(oxypeucedanin)	−	−	+	++	+++	−
6	(heraclenin)	−	+	++	++	+++	−
7	(oxypeucedanin hydrate)	−	+	++	+++	+++	−
10		−	−	+	+	++	−
11		−	−	−	+	++	−
13	(xanthotoxol)	−	−	−	−	−	−
14	(bergaptol)	−	−	−	−	−	−
15	(imperatorin)	−	−	+	+++	+++	−
16	(heraclenol)	−	−	+	++	+++	−
17		−	−	+	++	+++	−
18		−	−	+	++	++	−

[a] Compounds injected subcutaneously in 10 μL of Me_2SO solution over the skull cap (down removed). [b] Photosensitization responses were evaluated 6 days after a 24-h exposure of the treated chicks to long-wave ultraviolet light: (−) no response; (+) definite thickening of the skin; (++) skin blistered; (+++) severe phototoxicity characterized by darkened, dried, and hard skin. [c] See Table III for chemical names of compounds. [d] Compounds **4**, **8**, **9**, and **12** were not available in sufficient quantity for testing. [e] 10 μL of Me_2SO only. [f] Birds not exposed to UV light.

two isomers were prepared by reaction of the appropriate diol **7** or **16** with isovaleryl chloride. For the 8-substituted derivative, 200 mg of **16** was added to a 500-mL round-bottom flask containing 50 mL of dry ether and 100 mL of dry pyridine. Isovaleryl chloride (1.5 mL) was added dropwise to the stirred mixture, and after 30 min, the product was worked up by partitioning several times with 5% HCl to remove the pyridine. The ether was then concentrated to give an oily residue that was purified by TLC (solvent system 2), but could not be crystallized. The TLC purified oil (139 mg) showed identical TLC behavior and NMR and mass spectra to **11** isolated from the *A. majus* seed extract.

The 5-substituted isomer **10** was prepared in the same manner but on a much smaller scale (10 mg of starting diol **7**). The synthetic **10** (crystallized from ether–hexane) was identical in all respects (melting point, TLC, NMR, mass spectra) to **10** isolated from the plant extract.

Isomeric 5- and 8-[2-(Acetoxy)-3-hydroxy-3-methylbutoxy]psoralens (12 and 18). These isomers were synthesized by reacting the diols **7** and **16** with acetyl chloride by essentially the same procedure used to prepare the isovaleryl esters **10** and **11**. In the preparation of the 8-alkoxy ester **18**, 200 mg of the starting diol **16** yielded



18

only 37 mg of **18**. The product was purified on TLC (solvent system 2) but could not be crystallized. The structure of **18** was confirmed by NMR and mass spectral analysis (Tables III and IV). The 5-alkoxy ester **12** was prepared from 10 mg of **7**, also in low yield. The synthetic **12**, purified on TLC (solvent system 2) and crystallized from ether–hexane, was identical with **12** isolated from the *A. majus* seed extract on the basis of melting point, TLC, NMR, and mass spectra.

Occurrence of Synthetic Psoralens in *A. majus*. The synthetic psoralens **13**–**18** were compared by TLC in several solvent systems with components in the *A. majus* seed extracts. Long-wave UV was used to visualize all of the synthetic compounds except xanthotoxol and bergaptol, which were best visualized under short-wave UV. The comparisons revealed that none of the synthetic psoralens were present in detectable quantity in the plant extracts.

Phototoxicity. All of the *A. majus* psoralens that were available in sufficient quantity for testing were phototoxic to chick skin after subcutaneous injection and exposure of the birds to long-wave UV light (Table V). Although xanthotoxin, **1**, was possibly the most phototoxic of the compounds studied, other *A. majus* psoralens, including **2**, **6**, and **7**, elicited phototoxic responses at levels of injected compound as low as 3.3 μg. Each of the synthetic compounds tested was phototoxic at levels of 10 μg and above, with the exception of xanthotoxol (**13**) and bergaptol (**14**), which were completely inactive (Table V). The toxic symptoms observed in treated birds were in all cases dependent on exposure to long-wave UV. Chicks that were injected with the highest level of photosensitizer tested, but not exposed to long-wave UV, developed no toxic symptoms. Likewise, chicks injected with Me_2SO only and exposed to UV light did not show phototoxic effects (Table V).

DISCUSSION

The fact that eight of the twelve substituted psoralens isolated from *A. majus* seed in the current study have not previously been reported from this plant suggests that the psoralen chemistry of North American *A. majus* differs considerably from that of previously studied *A. majus* populations. At least eight psoralens occur in Egyptian or European *A. majus* (Abu-Mustafa et al., 1971, 1975a,b), and of these, **1**, **2**, **3**, and **4** were also isolated in the current study. At least 16 psoralens have now been obtained from *A. majus*; thus, this species is one of the richest known sources of linear furocoumarins.

Several of the *A. majus* psoralens are well-known and widely distributed plant products, but others are rare or previously unreported natural compounds. Two of the products, **8** and **10**, have each apparently been isolated from only one other plant source. The chlorohydrin **8**, isolated from *A. majus* seed in very low yield, was initially thought to have perhaps arisen as an artifact during the analysis procedure. However, careful TLC studies with

the purified 8 and both chloroform and acetone seed extracts indicated that 8 was indeed present in the *A. majus* seed. Compound 10, the isovaleric acid ester of the well-known plant psoralen oxypeucedanin hydrate (7), was isolated from the *A. majus* extracts in 0.1% yield, but its 8-substituted isomer 11 was obtained in much lower quantity (Table II). This report is apparently the first documentation of 11 and the related 12 as natural products.

Although the 8-substituted compound 1 was the major psoralen isolated from the seed extracts (Table II), eight of the twelve *A. majus* psoralens were 5-alkoxy derivatives, and one other compound, 3, was 5,8-disubstituted. The commercial availability of 1 made possible the synthesis of certain additional 8-alkoxy psoralens as TLC standards to determine their possible presence in the plant extracts. However, the synthetic 8-substituted analogues of the 5-substituted *A. majus* psoralens 4, 7, 8, and 12 (compounds 15, 16, 17, and 18, respectively) were not detected in TLC comparisons of the synthetic products with fluorescing components in the plant extracts. In spite of these findings, it seems almost certain that some of the 8-substituted psoralens synthesized were, in fact, present in minute amounts in the plant, or at least that they were generated as short-lived intermediates. Clearly, the presence of the 8-substituted compounds 6 and 11 in the plant necessitated 15 and 16 as respective intermediates. Imperatorin (16), although not seen as an *A. majus* constituent in the present study, has been isolated from Egyptian collections of the plant (Fahmy and Abu-Shady, 1948). Xanthotoxol (13) and bergaptol (14) have been reported from numerous plant sources and are logical intermediates in the biosynthesis of 8- and 5-alkoxy psoralens (Brown et al., 1970), but these alcohols were not detected in the plant extracts. However, 13 and 14 could only be visualized on TLC with short-wave UV and with relatively poor sensitivity; thus, their presence in the plant in low amounts would likely have gone undetected.

Each of the *A. majus* psoralens tested as photosensitizers in the chick bioassay system showed some degree of phototoxicity (Table V). The methoxy psoralens 1 and 2 were quite phototoxic, which was expected in view of numerous reports in the literature attesting to the high photosensitizing activity of these compounds. The relatively low phototoxicity of 3 in the chick bioassays was unexpected only in that others have reported 3 to be completely inactive as a photosensitizer in oral and dermal mammalian studies (Pathak and Fitzpatrick, 1959). The high phototoxicity of certain other *A. majus* psoralens was quite surprising, however, because most authors have found increasing chain length in the alkoxy psoralens to be accompanied by greatly reduced biological activity (Scott et al., 1976; Musajo and Rodighiero, 1972). In the current study, psoralens having rather large alkoxy or alkoxy ester substituents were in some cases quite phototoxic, and the most polar of the *A. majus* psoralens isolated, 7, was almost as phototoxic as 1 (Table V). It should be emphasized that the phototoxic effects reported here resulted from subcutaneous injection of chicks rather than oral or dermal exposure of mammals as is usually the case in psoralen bioassay. It may well be that the more polar psoralens penetrate skin poorly after dermal exposure and are likewise poorly absorbed from the digestive tract, or are rapidly detoxified, after oral exposure. Possible differences in susceptibility between birds and mammals also cannot be discounted.

These studies have shown that Texas *A. majus* contains a considerable number of phototoxic psoralens and that, at least in the ripened seed, the total psoralen content is quite high (Table II). Although the role of *A. majus* as a photosensitizing plant of range livestock in North America needs further clarification, these studies leave little doubt that consumption of the plant by livestock can be expected to cause phototoxic effects.

ACKNOWLEDGMENT

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Amino Acid Sequence of Roseotoxin B

George W. Engstrom

The amino acid sequence of roseotoxin B, a toxic cyclodepsipeptide isolated from ether extracts of *Trichothecium roseum*, has been determined by isolation and purification of peptides from partial acid hydrolysates and by complete acid hydrolysis and hydrazinolysis of the purified peptides. The hydroxy and amino acid sequence for the compound is cyclo-2-hydroxy-4-pentenoyl-*trans*-3-methylprolyl-L-isoleucyl-*N*-methylvalyl-β-alanyl-*N*-methylalanyl.

In 1975, Engstrom et al. reported that the identity of the one hydroxy and five amino acids found in the acid hydrolysate of roseotoxin B were 2-hydroxy-4-pentenoic acid, *trans*-3-methylproline, L-isoleucine, *N*-methylvaline, β-alanine, and *N*-methylalanine. This new toxic cyclodepsipeptide isolated from *Trichothecium roseum* had a molecular weight of 591 and an empirical formula of $C_{30}H_{49}O_7N_5$. The degree and nature of the toxicity of roseotoxin B in ducklings and mice has been reported previously by Richard et al. (1969, 1970).

The term "cyclodepsipeptide" was coined by Shemyakin (1960) and refers to cyclic compounds with alternating amino and hydroxy acid residues such as enniatin A (Quitt et al., 1963; Audhya and Russell, 1976), amino acids only such as telomycin (Sheehan et al., 1968), and those that consist of amino acids plus one hydroxy acid such as isariin (Vining and Taber, 1962), and esperin (Ito and Ogawa, 1959). Tamura et al. (1964) and Suzuki et al. (1966, 1970) have published structures of five cyclodepsipeptides produced by culture filtrates of *Metarrhizium anisopliae* (Mitchnikoff) Sorokin (formerly called *Oospora destructor*) which is a fungus that is pathogenic for insects. These compounds, called "destruxins", are similar in amino acid composition to roseotoxin B but were different in the hydroxy acid, the proline derivative, the valine derivative, or a combination of these. Cyclodepsipeptides have been isolated from bacteria, actinomycetes, and fungi. Several reviews on cyclodepsipeptides and related compounds have been published by Schröder and Lübke (1963), Losse and Bachmann (1964a,b), Russell (1966), Taylor (1970), Andreev et al. (1972), and Pressman (1977).

In this paper, new data concerning the chemical characterization of roseotoxin B are reported. Purification of the peptides from partial acid hydrolysates and identification of their hydroxy and amino acid residues have permitted formulation of the sequence of hydroxy and amino acids in roseotoxin B.

EXPERIMENTAL SECTION

Materials. The sources of chemicals and supplies were as follows: ninhydrin, L-proline, L-isoleucine, *N*-methyl-DL-alanine, *N*-methyl-DL-valine, from Sigma Chemical Co., St. Louis, Mo., β-alanine from Calbiochem-Behring Corp., San Diego, Calif.; hydrazine, fluorescamine from Pierce Chemical, Rockford, Ill.; isatin and zinc acetate from Fisher Scientific, Fair Lawn, N.J.; *trans*-3-methylproline from Dr. A. G. Mauger, Washington Hospital Center, Washington, D.C., and silica gel H and silica gel G manufactured by E. Merck AG Darmstadt, Germany, and distributed by Brinkmann Instruments, Des Plaines, Ill.

Methods. The purification procedure of roseotoxin B was described previously by Engstrom et al. (1975). Partial acid hydrolysis experiments involved dissolving crystalline roseotoxin B in concentrated HCl at room temperature (21–23 °C). Periods of hydrolysis include 1 and 3.5 days after which excess hydrochloric acid was removed by repeated flash evaporation (Buchler Instr., Fort Lee, N.J.). The partial acid hydrolysates were dissolved in small volumes of water for application to 20 × 20 cm thin-layer chromatograph (TLC) plates coated with 0.375 mm layer of silica gel H. The plates were air-dried overnight and activated by heating to 110 °C for 1 h before they were used. All the solvents were analytical reagent or reagent grade. All partial acid hydrolysates were chromatographed in order to isolate and identify the portion of each fraction that contained a peptide.

The first step in purification was TLC on silica gel H with ethyl acetate as the solvent. Silica gel from the upper half of each TLC plate was transferred to a flask, eluted with water, filtered through Whatman No. 42 filter paper, and flash evaporated to dryness. This was designated fraction I and contained a product from the partial acid hydrolysate that did not react with ninhydrin. This compound was designated peptide I although one of the residues was shown to be a hydroxy acid. The silica gel from the lower half of each TLC plate was processed in the same way and contained all the other fractions. This sample was applied to silica gel H plates which were then developed in the solvent mixture of benzene–ethanol–concentrated ammonium hydroxide (57:114:37, v/v/v). The plates were air-dried, and a narrow vertical band was scribed on both edges of each plate, sprayed with ninhydrin (Fahmy et al., 1961), and placed in an oven at 100 °C for 20 min to develop the color. The color bands that developed were used as the basis for scribing horizontal lines

The National Animal Disease Center, Science and Education Administration, Agricultural Research, U.S. Department of Agriculture, Ames, Iowa 50010.