AN INVESTIGATION OF THE ANTITUMOR ACTIVITY OF *MICROMELUM INTEGERRIMUM* (RUTACEAE)[1]

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ABSTRACT.—Extracts of *Micromelum integerrimum* (Buch.-Ham. ex Coleb.) M. Roem. were fractionated based on *in vivo* activity in mice in the P-388 lymphocytic leukemia system. Activity in ethanol extracts was concentrated in the chloroform partition fraction, which was further resolved by chromatography on silica gel. The known coumarins, micromelin and scopoletin, were crystallized from the active fractions and demonstrated to have antitumor activities. Micromelin was converted to the corresponding butenolide (deoxymicromelin) which was inactive in the 9KB assay.

Micromelum integerrimum (Buch.-Ham.) M. Roem. (Rutaceae) is a small evergreen tree indigenous to Asia (1). Initial collections from India of this species yielded extracts with cytotoxic activity against the *in vitro* 9KB human nasopharynx cell line (2). The 9KB activity was not reproducible on recollection. However, *in vivo* activity in mice against P-388 lymphocytic leukemia (2) was detected, and we undertook this study to fractionate the active extracts in order to isolate and identify the antitumor components. There has been no previous phytochemical work reported with this species; however, the coumarins, micromelin (micromelumin) and micropubescin, have been reported from *M. minutum* (Forst. f.) Seem (synonym: *M. pubescens* Blume) (3, 4).

After defatting with petroleum ether, a large portion of the stems and leaves of *M. integerrimum* was extracted with ethanol. The ethanol residue was partitioned between chloroform and water with the major P-388 activity (T/C 166% at 200 mg/kg) residing in the chloroform extract. Some initial activity (T/C 140% at 400 mg/kg) was detected in the lipoidal, petroleum ether residue; but only inactive fractions were obtained on subsequent extraction and column chromatography.

Column chromatography of the chloroform residue on silica gel eluted sequentially with benzene, benzene-chloroform, chloroform and methanol-chloroform yielded activity (T/C 130% at 20 mg/kg) in the chloroform and methanol-chloroform eluates. Extraction of the active eluates with ethyl ether followed by extraction of the ether residue in benzene-chloroform permitted crystallization of an active (T/C 149% at 10 mg/kg) component, compound **1**. A further portion of the chloroform partitioning residue was subjected to column chromatography on silica gel eluted with 0–10% increments of methanol in chloroform. By combining fractions on the basis of P-388 activity and tlc analysis, a second active component, compound **2**, was crystallized.

The uv absorptions at 221 and 318 nm, and ir absorption bands at 1730, 1630, and 1575 cm^{-1} of compound **1** suggested the coumarin framework, which was also compatible with the observation of eight sp^2 ^{13}C signals from 161–100 ppm (5). Further examination of the ^{13}C-^{1}H one-bond couplings indicated that the coumarin was disubstituted. The aromatic ^{1}H nmr resonance patterns (7.67 ppm, s, 1H; 7.08 ppm, s; 7.94 ppm, d, 9.5 Hz, 1H; and 6.26 ppm, d, 9.5 Hz) confirm this sub-

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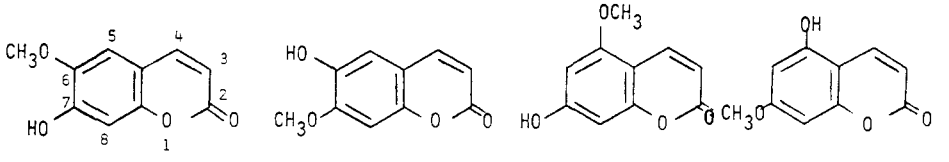
stitution pattern (6, 7). The presence of an aromatic methoxy positioned at C_7 was revealed by the ^{13}C nmr signals at 56.8 (OCH_3) and 100.1 (C_8) ppm and the ^1H nmr signals at 4.34 (OCH_3) and 7.08 (H_8) ppm. The lack of C_8–H_6 three bond coupling showed that the five-carbon substituent was attached at C_6 (5, 6). The existence of a trisubstituted epoxide was manifested by an unique ^{13}C signal at 62.8 ppm with large one-bond ^{13}C–^1H coupling constants (199 Hz) (8, 9). The ir absorption at 1785 cm^{-1} and the ^{13}C signal at 172.4 indicated the presence of a γ-lactone moiety. The ^1H and ^{13}C nmr spectra also suggested the presence of a methyl group (^1H:1.53 ppm, singlet, 3H and ^{13}C:10.8 ppm) and two methine groups (^1H:4.34 and 5.56 ppm, singlet and ^{13}C:6.28 and 77.8 ppm). On the basis of the spectral data, compound **1** was identified as 7-methoxy-6-[11,14-dihydro-15-methyl-12,13-epoxy-14-oxo-11-furanyl]coumarin, identical to the known compound, micromelin (3, 4). The lack of coupling between two aliphatic methine protons (H–11, H–12) is consistent with a *trans* relationship between these protons which results in an approximate 90° dihedral angle. Further confirmation of this structure was accomplished by extensive analysis of ^{13}C–^1H coupling patterns based on our previous ^{13}C nmr results (5) (table 1).

TABLE 1. *^{13}C Nmr chemical shifts (δ) and coupling constants (Hz) of micromelin* **1**, *in DMSO-d_6.*

Carbon	δ(ppm)	Multiplicity	J(Hδ)(coupled proton)
2	159.7	dd	11.6(H_4), 5.5(H_3)
3	113.2	d	174.0(H_3)
4	143.8	dd	166.6(H_4), 4.9(H_5)
5	129.5	dt	164.2(H_5), 4.3(H_4 and H_{11})
6	119.7	dd	5.8(H_8), 3.4(H_{11})
7	160.5	m	
8	100.1	d	164.8 (H_8)
9	156.2	dt	9.8(H_5), 5.8(H_4 and H_5)
10	111.9	ddd	8.1(H_3), 4.7(H_5), 1.4(H_4 or H_5)
11	77.8	ddd	156.9(H_{11}), 11.1(H_{12}), 5.0(H_5)
12	62.8	ddq	199.0(H_6), 5.9(H_{12}), 3.1(H_{15})
13	57.7	qd	6.1(H_{15}), 2.8(H_{11} or H_{12})
14	172.4	dq	6.1(H_{11}), 2.9(H_{15})
15	10.8	q	129.2(H_{15})
16	56.8	q	146.5(H_{16})

The uv and ir spectra data of compound **2** indicated the basic skeleton of an hydroxycoumarin. The existence of a methoxy group was shown by the ir absorption at 1375 cm^{-1} and the ^1H nmr signal at 3.92 ppm (s, 3H). The ^1H nmr spectral pattern suggested that this compound was either scopoletin or isoscopoletin (6, 10, 11). The other alternative structures (**4** and **5**) were excluded because both H_6 and H_8 would be expected to resonate at a higher field (<6.4 ppm) in **4** and **5**. The final identification of compound **2**, as scopoletin, was achieved by comparison (mp, ms, ir, uv, ^1H nmr) with reported physical properties (10, 11) and with authentic samples of scopoletin and isoscopoletin.



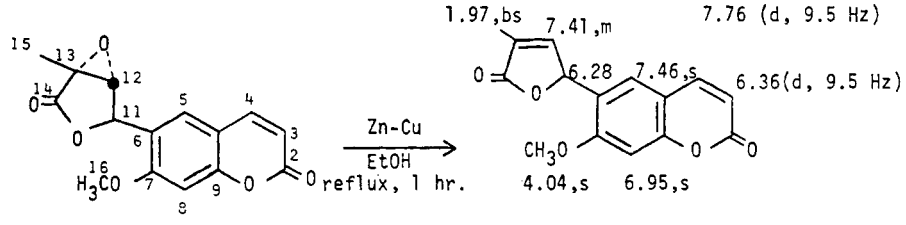
2 **3** **4** **5**

TABLE 2. *Physical properties of compound* **2**, *scopoletin, and isoscopoletin.*

	Compound **2**	Scopoletin (11)	Isoscopoletin (10, 11)
mp	208–209° C	203–205° C	184–186° C
ms	192	192	192
ir	3330, 1690, 1600, 1555, 1500, 1435, 1375, 1280		3500–3300, 1700, 1620, 1552
uv	345 (log ϵ=4.31)	350	349
	297 (3.94)	292	292
	254 (3.88)	260	258
	228 (4.36)	225	227
^{1}H nmr[a]	7.63 (1H, d, 9.5 Hz)	7.8 (1H, d, 10 Hz)	7.61 (1H, d, 10 Hz)
	6.90 (1H, s)	7.13 (1H, s)	6.91 (1H, s)
	6.86 (1H, s)	6.72 (1H, s)	6.78 (1H, s)
	6.20 (1H, d, 9.5 Hz)	6.10 (1H, d, 10 Hz)	6.14 (1H, d, 10 Hz)
	3.92 (3H, s)	3.80 (3H, s)	3.91 (3H, s)

[a]Solvents: $CDCl_3$+DMSO-d_6 (Compound **2** and Isoscopoletin); and DMSO-d_6 (Scopoletin).

Compound **1**, micromelin (NSC–281267), initially demonstrated significant P–388 activity (T/C 149% at 10 mg/kg) but was inactive in lower doses and in subsequent tests. Micromelin has also shown significant but erratic activity (T/C 228% at 1.25 mg/kg) against the Lewis lung carcinoma; further testing is in progress (2). Micromelin was converted by Zn-Cu (13) to 12,13-deoxymicromelin, **6**; this derivative, like **1**, was inactive in the 9KB assay, and insufficient compound was obtained for *in vivo* evaluation. Examination of NCI testing files showed that compound **2**, scopoletin (NSC 405647) had sporadic P–388 activity (T/C 130% at 400 mg/kg) but this activity was variable; it was inactive in the 9KB, L–1210 lymphoid leukemia (LE), and sarcoma 180 (SA) assays, and was also inactive (T/C 48% at 100 mg/kg) in the adenocarcinoma 755 (CA) test (2). Solubility problems in the vehicles used for testing may be a factor in the sporadic activity of **1** and **2**.



1 **6**

EXPERIMENTAL[3]

[3]The ir spectra were taken in KBr on a Beckman 112 4230 spectrophotometer. Uv spectra were obtained on a Cary model 17 recording spectrometer. The nmr spectra were recorded on Varian EM 360 (^{1}H nmr) and Jeol PFT–100 (^{13}C nmr) spectrometers utilizing TMS (^{1}H nmr) and deuteriodimethyl sulfoxide (^{13}C nmr) as the internal standards. The nmr shifts are reported in δ(ppm) units, $\delta(^{13}C)$ (TMS): δ(DMSO-d_6)+39.6 ppm. The low resolution ms were obtained on a DuPont model 21–492B spectrometer. Silic AR CC–7 (Mallinckrodt) was used for column chromatography, and silica gel 60 F254 (EM Reagent) was used for preparative tlc. All mps are uncorrected. The 9KB cytotoxicity assays were performed at the Cell Culture Laboratory, Purdue Cancer Center; other antitumor assays were performed at Raltech, Madison, Wisconsin.

PLANT MATERIALS.—Dried stem-leaf and wood-stem materials of *Micromelum integerrimum* (Buch.-Ham. ex Coleb.) M. Roem. (B-630897, PR-45705 and PR-45756) were collected in India and authenticated by the Medicinal Plant Resources Laboratory, USDA, Beltsville, Maryland, through which voucher specimens are preserved. The material was pulverized in a Fitzpatrick mill. The wood-stem material was inactive in the initial screens and was not examined.

EXTRACTION AND FRACTIONATION.—Finely ground stem-leaf material (887 g) of *M. integerrimum* was defatted with petroleum ether in a Soxhlet apparatus then exhaustively extracted with 95% ethanol in a percolator. After the ethanol was removed under reduced pressure, the residue was partitioned between $CHCl_3$ and water (1:1); the residues showed P-388 activities of T/C 166% at 200 mg/kg ($CHCl_3$) and 149% at 100 mg/kg (H_2O). A 2.5 g portion (from 14.2 g) of the $CHCl_3$ was subjected to column chromatography on 150 g of silica gel eluted sequentially with benzene, benzene: $CHCl_3$ (2:1, 1:1, 1:2), $CHCl_3$, and MeOH-$CHCl_3$ (1:99, 1:49). Activity (T/C 130% at 20 mg/kg) was concentrated in the $CHCl_3$ and MeOH-$CHCl_3$ (1:99).

ISOLATION AND IDENTIFICATION OF MICROMELIN, **1**.—A portion of the residue from the active fraction (150 mg of 306 mg) was washed with ethyl ether (20 ml), and the residue from the soluble material was taken up in benzene: $CHCl_3$ (1:1, 10 ml). On concentration and storing at room temperature, crystals of **1** (30 mg, 0.04% yield) were formed and were recrystallized from $CHCl_3$ as colorless needles; mp 216–218°; ms (*m/e*): 288 (M^+), 229, 213, 186, and 158; λ max (MeOH) 221 (log ε=3.99), 240(3.80), and 250(3.55), 292(3.99) and 318(4.2;) nm; ir (KBr): 3040, 1785, 1730, 1630, 1573, 1277, 923, and 830 cm^{-1}; ^1H nmr (DMSO-d_6): 1.61(s, 3H), 3.96(s, 3H), 4.42(s, 1H), 5.64(s, 1H), 6.34(d, 9.5 Hz, 1H), 7.16(s, 1H), 7.75(s, 1H), and 8.02(d, 9.5 Hz, 1H); ^{13}C nmr (see Table 1). This compound was identified as micromelin (lit. mp 218–219°) (3, 4).

ISOLATION AND IDENTIFICATION OF SCOPOLETIN, **2**.—A portion of a $CHCl_3$ residue from a larger batch of stem-leaf extract (87.5 g from 2.7 kg of plant material) was processed in order to isolate additional micromelin, **1**. P-388 activity (e.g., T/C 138% at 15 mg/kg) was detected in additional column fractions which, as monitored by tlc ($CHCl_3$-MeOH, 50:1, on silica gel), were lacking in micromelin. The major component of these fractions was isolated as follows. The $CHCl_3$ partitioning residue (20 g) was washed with petroleum ether, and the insoluble material (11.9 g) was subjected to column chromatography on silica gel (150 g) eluted with increments of 0–10% methanol in $CHCl_3$. The major $CHCl_3$ eluate (1.478 g) contained micromelin and another component (tlc). A portion (1.35 g) of this fraction was washed with petroleum ether-benzene (1:1, 20 ml), and the insoluble material (930 mg) was again subjected to column chromatography on silica gel (130 g) eluted with $CHCl_3$. A large amount of micromelin (588 mg) was isolated from the early fractions, but two later fractions were free of other materials and yielded, on recrystallization from $CHCl_3$, 17 mg (0.0006% yield) of colorless needles of compound **2**: mp 206–207°; ms (*m/e*): 192 (M^+), 177, 164, 149, 121, 79, and 69; uv λ max (MeOH) 228 (log ε=4.36), 254(3.88), 297(3.94), and 345(4.31) nm; ir (KBr): 3330, 1690, 1555, 1280, and 1135 cm^{-1}; ^1H nmr (see Table 2). On the basis of the data the compound was identified as scopoletin (lit. mp 203–205°) (10).

CONVERSION OF MICROMELIN **1**, TO DEOXYMICROMELIN **6**.—A mixture of micromelin (75 mg) and zinc-copper couple (2 g) in ethanol (70 ml) was refluxed for 1 hr (12). The filtrate was evaporated under reduced pressure to give a white residue which was extracted with $CHCl_3$. The extract was dried over anhydrous Na_2SO_4 and condensed to dryness under reduced pressure. The residue was purified by preparative tlc ($CHCl_3$-ethyl ether-n-hexane, 2:2:1) on silica gel. Recrystallization from chloroform yielded 33 mg of needles (44%), mp 224–226°; ms (*m/e*): 272 (M^+), 243, and 203; uv λ max (MeOH): 221 (log ε=4.22), 245(3.62), 256(3.55), and 332(4.20) nm; ir (KBr): 1750, 1730, 1620, 1130, and 1010 cm^{-1}; ^1H nmr (see illustration of **6**).

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