Antitumor Agents I: Angustibalin, a New Cytotoxic Sesquiterpene Lactone from *Balduina angustifolia* (Pursh.) Robins

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Abstract □ A chloroform extract of the whole plant of *Balduina angustifolia* showed significant *in vitro* inhibitory activity when tested against the growth of tissue culture cells derived from human epidermoid carcinoma of larynx (H.Ep.-2). The extract was examined and found to contain three known compounds. These included rotenone and helenalin, two cytotoxic constituents, and hispidulin, an inactive flavone. The chloroform extract also contained a new cytotoxic pseudoguaianolide sesquiterpene lactone, provisionally named *angustibalin*, which was identified as the acetate of helenalin. The relatively low content (0.024%) of helenalin in comparison with the previously reported high yield (0.53%) is noted. The isolation of rotenone also provided the first instance for its occurrence from the Compositae family.

Keyphrases □ *Balduina angustifolia* (Pursh.) Robins—isolation, identification, of four cytotoxic constituents □ Angustibalin—isolation from *Balduina angustifolia*, structure identification, cytotoxic activity □ Rotenone—isolation from *Balduina angustifolia*, cytotoxic activity □ Antitumor agents, potential—constituents of *Balduina angustifolia* □ Cytotoxicity—constituents of *Balduina angustifolia* □ Structure-activity relationships—sesquiterpene lactones as antitumor agents

Examination of the helenalin-related sesquiterpene lactonic content of the whole plant of *Balduina angustifolia* (Pursh.) Robins[1] was a continuation of a general program (1) for investigation of the relationship between the sesquiterpene lactonic structure and the antitumor or cytotoxic activity. Of further interest was the fact that the chloroform extract of this particular species was found to show reproducible inhibitory activity against the cell culture of a human epidermoid carcinoma of larynx (H.Ep.-2)[2].

The extraction of the active principles was carried out according to an exact procedure described in the literature (2). The chloroform extract was column chromatographed over silica gel to yield fractions containing mixtures of four crystalline substances. These substances were tested for cytotoxicity against three different cell lines originating from normal human fibroblasts, human laryngeal carcinoma, and human cells transformed with simian virus 40. The results are presented in Table I.

DISCUSSION

Angustibalin (II), the new cytotoxic principle, was isolated from the chloroform eluate. The compound, m.p. 181°, $[\alpha]_D^{25}$ −54°, has the composition $C_{17}H_{20}O_5$ and showed prominent peaks in the mass spectrum at *m/e* 262, 124 (base peak), 123, 96, and 95. Angustibalin revealed a UV maximum at 221 nm. (log ε 4.12), IR bands at 1768 and 1658 cm.$^{-1}$, and a pair of low field doublets in the NMR spectrum at δ 6.08 (1H, J = 3) and 6.38 (1H, J = 3), characteristic of a γ-lactone conjugated with an exocyclic methylene grouping, a feature common to many sesquiterpene lactones of Compositae (4). An IR band at 1733 cm.$^{-1}$ suggested the presence of an acetoxy grouping, and an NMR signal at δ 1.99 (3H, singlet) and a mass spectral peak at *m/e* 262 (M-42) (M—CH_2=C=O) established the presence of this structural feature. The presence of a cyclopentenone ring system in angustibalin was first indicated by the appearance of an IR band at 1710 cm.$^{-1}$ and was substantiated by the presence in the NMR spectrum of a pair of typical *AB*-type low field doublets at δ 7.64 (1H, dd, J = 6, 2.3, H_2) and 6.10 (1H, overlapped dd, J = 6, 2.3, H_3). The NMR spectrum of angustibalin also exhibited a broad one-proton singlet at δ 5.35 attributable to H_6 and a one-proton triplet at 4.87 (dt, J= 6.8, 2.3) which was assigned to the lactonic proton, H_8. The remaining sharp singlet at 1.02 (3H) and doublet at 1.27 (3H, J = 6) were designated for C_5—CH_3 and C_{10}—CH_3, respectively.

The foregoing evidence leads to the assignment of Structure II for angustibalin. To confirm further the structure of angustibalin, I was established by synthesizing this compound from helenalin (1). The synthetic product, the acetate of helenalin, obtained by acetylation of helenalin with pyridine in acetic anhydride was found to be identical with angustibalin by direct IR, NMR, and mass spectroscopic comparison and mixed melting-point determination. The acetate of helenalin was also prepared previously (5–7) during structural elucidation of the naturally occurring helenalin.

The initial chloroform eluate also provided a potent cytotoxic substance, I. Compound I, $C_{23}H_{22}O_6$, m.p. 164°, $[\alpha]_D^{25}$ −225.6°, shows a molecular ion peak at *m/e* 394 and the other prominent peaks in the mass spectrum at *m/e* 379 (M-15), 192 (M-192) (base peak), and 177 (M-192-15). The loss of the fragment of 192 unit is suggestive of a retro-Diels-Alder ring fission, a diagnostic fragmentation pattern for the mass spectra of rotenoids (8, 9) as shown in Scheme I[3].

The NMR spectrum[4] of Compound I displayed signals at δ 1.77 (3H, s, vinyl methyl), 3.76 (3H, s), 3.80 (3H, s) (two OCH_3), 6.48 (1H, overlapped s, H_4), 6.82 (1H, s, H_1), and a pair of doublets at 6.55 (1H, J = 9, H_{10}) and 7.93 (1H, J = 9, H_{11}), respectively. The identity of I was established by direct comparison with an authentic sample of rotenone[5], a principal insecticidal constituent of derris root, cubé, *etc.* (Leguminosae) (11).

The final chloroform fractions also afforded a cytotoxic substance, helenalin (III), $C_{15}H_{18}O_4$, m.p. 170–172°, which showed an IR spectrum at 3340 (OH), 1754 (γ-lactone), and 1700 cm.$^{-1}$ (cyclopentenone), and NMR signals at δ 0.98 (3H, s, C_5—CH_3), 1.28 (3H, d, J = 6, C_{10} — CH_3), 4.45 (1H, d, J = 4.5, H_6), 5.00 (1H, t, J = 6, H_8), 5.85 (1H, d, J = 3, H_{13}), 6.40 (1H, d, J = 3, H_{13}), 6.09 (1H, dd, J = 6, 3, H_3), and 7.77 (1H, dd, J = 6, 1.5, H_2). The correspondence of the properties of this compound with those described in the literature (1, 3, 5, 6, 12–14) and its conversion into angustibalin by acetylation established its identity.

The inactive flavone (IV), m.p. 292°, was isolated from chloroform–acetone fractions. It gives a red color with Mg and HCl and an olive-green color with $FeCl_3$. Its mass spectrum and elemental

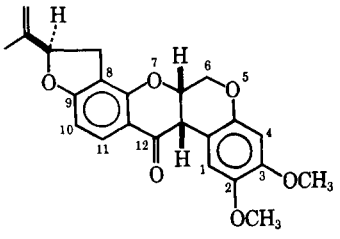
[1] This plant is also known as *Actinospermum angustifolium* as reported in the literature (3).

[2] The cell culture assay was performed according to test procedures described in the literature (1).

[3] The m* indicates that the proposed transition is supported by a metastable ion.

[4] The NMR spectrum of rotenone was reported previously by Crombie and Lown (10), determined at 56.445 MHz. on a Varian Associates NMR spectrometer (model V. 4300). They reported a singlet at δ 3.76 (6H) for two OCH_3 groups and the other signals at δ 6.68 (1H, s, H_1), 6.41 (1H, s, H_4), 6.47 (1H, d, J = 11, H_{10}), 7.74 (1H, d, J = 11, H_{11}), and 1.75 (3H, s, C—CH_3).

[5] Marketed by Aldrich Chemical Co., Inc.



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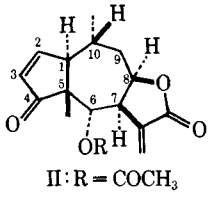
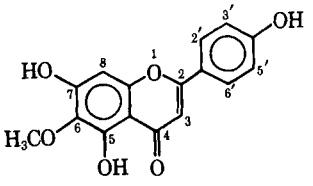


II: R = $COCH_3$

III: R = H



IV

analysis, which corresponded to the formula $C_{16}H_{12}O_6$, along with the color reactions and IR and UV spectra suggested that the compound was a monomethoxytrihydroxy flavone. The NMR spectrum (measured in acetone-d_6 with traces of dimethyl sulfoxide-d_6) showed one three-proton singlet at δ 3.86 (OCH_3), two one-proton singlets at δ 6.65 and 6.68 (H_3 and H_8), and an A_2B_2-type coupling pattern at δ 7.98 (2H, d, J = 8) and 7.05 (2H, d, J = 8), which were ascribed to the aromatic protons at $H_{2',6'}$ and $H_{3',5'}$, respectively.

The evidence is indicative of Structure IV (*i.e.*, 4′,5,7-trihydroxy-6-methoxyflavone) for the flavone. Added confirmation was obtained when Compound IV was compared with an authentic sample of hispidulin isolated from *Ambrosia hispida* Pursh. (15) and from *Helenium microcephalum* M. A. Curt. ex Gray[6].

The basic structural requirement for the high level of cytotoxicity of angustibalin (II) and helenalin (III) is due mainly to the introduction of the O=C—C=CH_2 system. This grouping is found in both compounds as an α-methylene-γ-lactone moiety and an α,β-unsaturated ketone system, as previously reported (1).

EXPERIMENTAL[7]

Extraction of *Balduina angustifolia* (Pursh.) Robins[8]—The *B. angustifolia* (Compositae) used was from a collection made in Florida during September 1964. The ground, air-dried, whole plant material (18 lb.) was exhaustively extracted with chloroform at room temperature, yielding, after removal of the solvent, a thick green-black tar. This was shaken with a mixture of methanol (1.2 l.), hexane (1.2 l.), and water (400 ml.). The aqueous layer was washed well with hexane, and the hexane layer was reextracted with water. The combined aqueous extracts were concentrated somewhat *in vacuo* and extracted with chloroform. The chloroform extract, upon evaporation, yielded 117.5 g. of a dark-brown syrup. TLC showed that this crude extract was a mixture of four components.

Isolation of Rotenone, Angustibalin, Helenalin, and Hispidulin—The crude residue was chromatographed on silica gel (800 g.), with elution with chloroform, chloroform–acetone, and acetone. Sixty-six fractions of about 300 ml. each were collected and examined by TLC. The first chloroform eluate (Fractions 1–3) contained only traces of low melting waxes. The subsequent chloroform eluate (Fractions 4–7) contained mainly material giving a single fast moving spot on TLC. Fractions 8–15 (chloroform) afforded a mixture of three components. A mixture of two components was also obtained from the final chloroform eluate (Fractions 16–46). The chloroform–acetone (1:1) eluate (Fractions 47–52) gave a single orange-yellow spot on TLC, together with a mixture of other polar compounds. Fractions 53–66 (acetone) yielded a mixture of dark-brown oily polar substances from which no satisfactory compounds could be obtained.

Table I—Cytotoxicity of Constituents from *B. angustifolia*

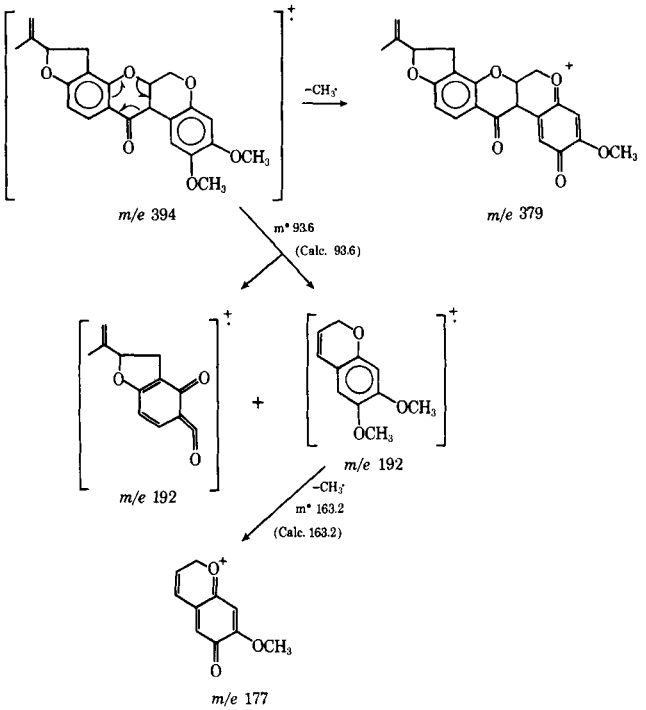
Number	Compounds	ED_{50}, mcg./ml.[a] WI-38[b]	H.Ep.-2[c]	W-18 Va-2[d]
I	Rotenone	0.05	0.06	0.04
II	Angustibalin	0.08	0.29	0.07
III	Helenalin	0.03	0.08	0.07
IV	Hispidulin	31.90	>40	26.70

[a] ED_{50} values were determined based upon the rapid microtiter method described (1). [b] WI-38 refers to normal human diploid fibroblasts. [c] H.Ep.-2 refers to human epidermoid carcinoma of larynx. [d] W-18 Va-2 refers to simian virus 40-transformed cells of human origin.

Rotenone (I)—The brown syrup obtained from Fractions 4–7 yielded crystals (2.8 g.) upon addition of ether–dichloromethane (1:1). Two recrystallizations of this compound (I) from dichloromethane yielded fine white needles (2 g.), m.p. 164°; $[\alpha]_D^{25}$ − 225.6° (c, 0.5, benzene); IR $\nu_{max.}$ cm.$^{-1}$: 1678, 1670 (conjugated C=O), 1610, 1593, 1520 (aromatic C=C), 1650, and 915 (terminal methylene); UV $\lambda_{max.}$ nm. (log ϵ): 212 (4.47), 220 (shoulder) (4.39), 237 (4.16), 244 (shoulder) (4.12), and 295 (4.23). A mixed melting point with authentic rotenone[5] showed no depression, and the IR spectra were identical.

Anal.—Calc. for $C_{23}H_{22}O_6$: C, 70.04; H, 5.62. Found: C, 70.36; H, 5.76.

Angustibalin (II)—The dark-brown syrup resulting from Fractions 8–15 was rechromatographed on silica gel (75 g.) and eluted with petroleum ether–chloroform (3:4). Eighty-five 10-ml. fractions



Scheme I—The metastable ion (m) at 93.6 was not observed previously (8, 9).*

[6] T. A. Geissman and K.-H. Lee, unpublished data.

[7] Unless otherwise specified, melting points were determined on a Thomas-Hoover melting-point apparatus and are corrected. Optical rotations were determined on a Perkin-Elmer model 141 polarimeter. UV spectra were taken in ethanol solutions with a Cary model 15 spectrophotometer. IR spectra were determined in mineral oil mulls with a Perkin-Elmer 257 grating IR spectrophotometer. NMR spectra were measured in $CDCl_3$ with a Jeolco C 60 HL NMR spectrometer using tetramethylsilane as an internal standard; s refers to singlet, d to doublet, and t to triplet, and the J values are in hertz. Mass spectra were determined on a Hitachi RMU-7 instrument at 70 ev. using a direct inlet system. Silica gel for column chromatography refers to Baker A.R. No. 3405, and silica gel for TLC refers to Merck silica gel G developed with petroleum ether–chloroform (3:4) and visualized with iodine vapor. Elemental analyses were performed by Atlantic Microlab, Inc., Atlanta, Ga.

[8] The authors thank Dr. M. E. Wall and Mr. H. L. Taylor, Research Triangle Institute, N. C., for providing the plant material (Batch No. RT4147), and Dr. R. E. Perdue, Jr., of the U. S. Department of Agriculture, Beltsville, Md., for providing the identification number (No. PR 8878) of the plant material.

were collected. A mixture of two components (I and II) was obtained from Fractions 1–37. The subsequent fractions (38–78) yielded the new sesquiterpene lactone angustibalin (II), and the later fractions (79–85) yielded a mixture of two compounds (II and III). The orange-colored oily substance obtained from Fractions 38–78 crystallized upon addition of dichloromethane. Two recrystallizations from dichloromethane afforded colorless needles (600 mg.), m.p. 181°, $[\alpha]_D^{25}$ −54° (c, 0.1). The relevant spectral (UV, IR, NMR, and mass) characteristics have been described in the text. Angustibalin failed to depress the melting point of an authentic sample of acetylhelenalin (*q.v.*) on admixture, and the IR, NMR, and mass spectra were identical.

Anal.—Calc. for $C_{17}H_{20}O_5$: C, 67.09; H, 6.62. Found: C, 66.94; H, 6.71.

Helenalin (III)—The brownish syrup from Fractions 16–46 was added with ether, and the crystalline material (2 g.) that formed was twice recrystallized from benzene to give colorless needles, m.p. 170–172°. The identity of this compound with an authentic sample of helenalin (1) was established by TLC, IR, and NMR spectroscopic comparison and mixed melting-point determination.

Hispidulin (IV)—The yellowish solids obtained from Fractions 47–52 after evaporation of solvent were twice recrystallized from acetone to yield yellow crystals (300 mg.), m.p. 292°; IR $\nu_{max.}$ cm.$^{-1}$: 3400, 3330 (broad, bonded OH), 1650 (conjugated, hydrogen-bonded C=O), 1605, 1590, 1580, and 1550 (aromatic C=C); UV $\lambda_{max.}$ nm. (log ϵ): 217 (4.45), 275 (4.26), and 338 (4.44); mass spectrum, *m/e:* 300 (M^+) (base peak), 285 (M-15), and 257 (M-15-28). The NMR spectrum has been described. The melting point reported for hispidulin is 291–292° (methanol) (15). The identity of this compound with an authentic sample of hispidulin[6] was confirmed by mixed melting point, TLC, and superimposable IR spectra.

Anal.—Calc. for $C_{16}H_{12}O_6$: C, 64.00; H, 4.03. Found: C, 64.32; H, 3.93.

Synthesis of Helenalin Acetate—A solution of helenalin[9] (100 mg.) with acetic anhydride (0.5 ml.) in anhydrous pyridine (1 ml.) was allowed to stand at room temperature overnight. The reaction mixture was diluted with water and extracted with chloroform. The chloroform solution was washed with 10% aqueous hydrochloric acid (5 ml.) and water, dried, and evaporated under reduced pressure to furnish the quantitative yield of helenalin acetate as colorless crystals. Two recrystallizations from ether–methanol yielded fine colorless needles, m.p. 180–181° [lit. m.p. of acetyl helenalin 184° (aqueous methanol) (5) and 179.5–180.5° (aqueous methanol) (6)]. The IR spectrum of this compound was identical with that of angustibalin as already described.

[9] Obtained from *H. microcephalum* as reported in the literature (1).

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