# ANTITUMOR AGENTS. 49.[1] TRICIN, KAEMPFEROL-3-O-β-D-GLUCOPYRANOSIDE AND (+)-NORTRACHELOGENIN, ANTILEUKEMIC PRINCIPLES FROM *WIKSTROEMIA INDICA*

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ABSTRACT.—Bioassay-directed isolation of the antitumor extract of *Wikstroemia indica* (Thymelaeaceae) has led to the characterization of tricin, kaempferol-3-*O*-β-D-glucopyranoside, and (+)-nortrachelogenin as the major antileukemic constituents. In addition, daphnoretin was identified as the potent antitumor principle *in vivo* against the Ehrlich ascites carcinoma growth in mice.

The whole plant of *Wikstroemia indica* C. A. Mey (Thymelaeaceae), known as "Nan-Ling-Jao-Hua" or "Po-Lun" in Chinese folklore, is used as a herbal remedy for the treatment of human syphilis, arthritis, whooping cough (1), and cancer (2). (+)-Nortrachelogenin, a CNS depressant, and daphnoretin have recently been isolated from *W. indica* (3). However, no report on the isolation and characterization of antitumor constituents from this plant has been made, although isolation of wikstromol, an antitumor lignan, has been reported in other related species, *Wikstroemia foetida* var. *oahuensis* and *Wikstroemia uva-ursi* (4). As a result of a continued search among Chinese plants for new and novel naturally occurring potential antitumor agents (5, 6), the methanolic extract of the stems of *W. indica* was found to show significant inhibitory activity *in vivo* against the Ehrlich ascites carcinoma growth in mice (97% inhibition) as well as the P-388 lymphocytic leukemia growth in mice (T/C = 180%) at 50 mg/kg/day, I.P. Guided by both the antineoplastic *in vivo* Ehrlich ascites carcinoma[2] (7) and *in vivo* P-388 lymphocytic leukemia[3] (8) assays, the isolation of active principles was carried out according to a procedure described previously (9). Column chromatography of the active final chloroform extract on silica gel led to the isolation of antitumor constituents **1**, **2**, **3** and **4** in 0.03%, 0.0038%, 0.0031% and 0.0015% yields, respectively.

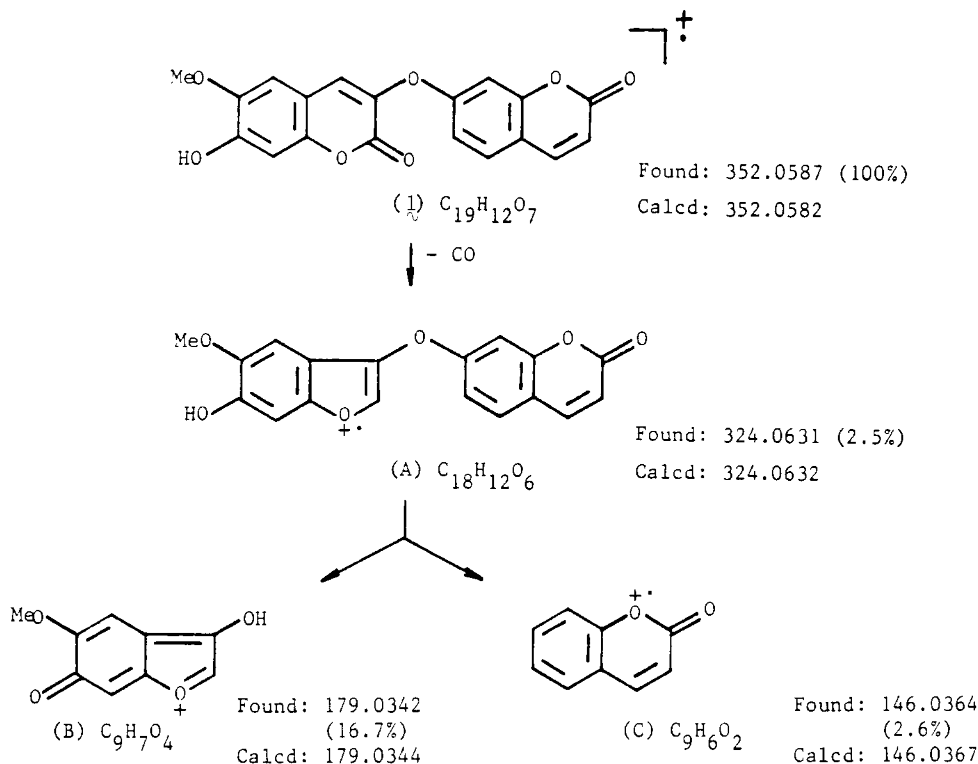
Compound **1** [pale yellow needles, mp 246–246° *m/z* 352.0579 ($M^+$), base peak] had a molecular formula $C_{19}H_{12}O_7$. Compound **1** exhibited a characteristic uv spectrum [λ max nm (log ε) at 228 (4.19), 264 (3.95), 325 (4.23) and 342 (4.27)]

[1]For part 48 see Y. F. Liou, I. H. Hall, M. Okano, K. H. Lee and S. G. Chaney, *J. Pharm. Sci.*, in press.

[2]Anticarcinoma activity was assayed in $CF_1$ male mice (~25g). Eighty percent inhibition of tumor growth is required for potent activity.

[3]Antileukemic activity was assayed according to a literature method (8) in $BDF_1$ male mice (~20g). A compound is active if it exhibits a T/C of ~ 120% (8).

due to the presence of the coumarin nucleus. This was further confirmed by a mass peak at *m/z* 324 (A) which resulted from the loss of CO as seen in a typical fragmentation pathway of coumarins (10). The peaks at *m/z* 179 (B) and 146 (C) arising from the cleavage of A, as shown below, was indicative of the presence of a dicoumaryl ether for the structure of **1**.

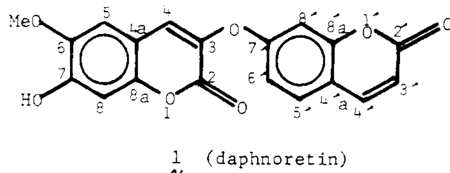



Added confirmation was obtained when compound **1** was directly compared (tlc and uv, ir and pmr spectra) with an authentic sample of daphnoretin isolated from *Daphne mezereum* (11).

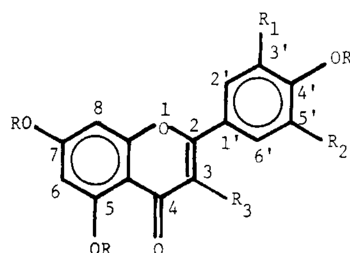
Compound **2**, mp 276–279°, was assigned the structure 5,7,4'-trihydroxy-3', 5'-dimethoxyflavone (i.e. tricin) (12, 13) on the basis of the following spectral and chemical evidence. It was analyzed for $C_{17}H_{14}O_7$ ($M^+$, 330.0732). Its uv spectrum revealed the presence of a flavone skeleton [λ max (MeOH) at 268 and 348 nm] (14). Its $^1$H-nmr spectrum indicated that the 3,6,8,2' and 6'-positions in the flavone skeleton were unsubstituted (15) as protons at C–6 and C–8 appeared separately as doublets (d, $J=2.5$ Hz) at δ 6.29 and 6.59, respectively. The C–3 proton in **2** was seen as a sharp singlet at δ 6.77. The singlet for two protons at δ 7.42 suggested a 3',4',5'-trioxygenated pattern in which H–2' and H–6' were equivalent. The dimethoxyl groups were placed at the 3' and 5' positions as they appeared as a six-proton singlet at δ 3.98. The hydroxyl groups were located at δ 13.04 (1H, s, OH–5) and 3.80 (2H, br.s, OH–5 and –7). The mass spectrum of **2** displayed common fragment ions which were in agreement with the 5,7,4'-trihydroxy-3',5'-dimethoxyflavone structure. Acetylation of **2** with acetic anhydride in pyridine under reflux gave rise to a triacetate **2a** (mp 249–250°; lit. 13 reported mp 251–254°) whose spectral data were in accord with the assigned

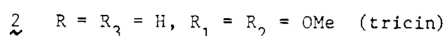
structure. The confirmation of the structure of **2** was made by a direct comparison with an authentic sample of tricin (12).

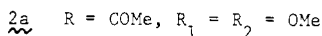
Compound **3** (yellow needles, mp 235–237°), $[\alpha]^{25}D -60°$, gave, on treatment with acetic anhydride in pyridine, a hepta acetate (**3a**, mp 214–215°, $C_{35}H_{34}O_{18}$). Acid hydrolysis of **3** with 1:1 hydrochloric acid-methanol yielded an aglycon

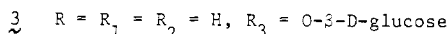



1 (daphnoretin)

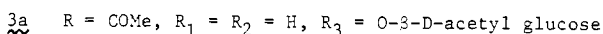


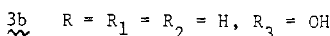


2 R = R3 = H, R1 = R2 = OMe (tricin)

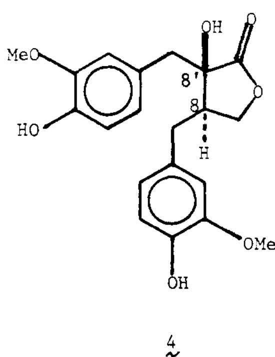

2a R = COMe, R1 = R2 = OMe

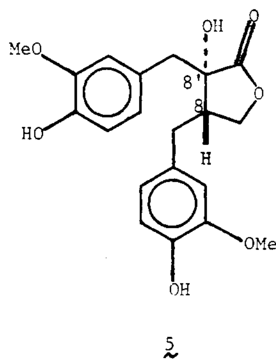

3 R = R1 = R2 = H, R3 = O-β-D-glucose

(kaempferol-3-O-β-D-glucopyranoside)


3a R = COMe, R1 = R2 = H, R3 = O-β-D-acetyl glucose


3b R = R1 = R2 = H, R3 = OH




4
[(+)-nortrachelogenin (wikstromol)] - 8(R)8'(R)

5
[(-)-nortrachelogenin] - 8(S)8'(S)

[**3b** mp 274–276° (dec.), $C_{15}H_{10}O_6$] identical to a sample of kaempferol.[4] Final identification of **3** as kaempferol 3-*O*-β-D-glucopyranoside was also obtained by direct comparison (undepressed mixed mp and identical ir, nmr and mass spectra) with an authentic specimen isolated from *Pinus contorta* (16).

Isolation of other known active components from this extract included (+)-nortrachelogenin (**4**),[5] whose structure was identified by direct comparison (mixture mp, ir and nmr spectra) with its corresponding authentic specimen (3).

Although daphnoretin (**1**) was found to be inactive as an antileukemic agent in the *in vivo* P–388 screen as also reported previously (4), we found that this compound caused 97% inhibition of Ehrlich ascites cell growth at 3 mg/kg/day I.P. Further studies on the mechanisms of action of **1** are in progress.

Tricin (**2**) afforded a T/C=133 and 174% when tested at 6 mg/kg and 12.5 mg/kg, respectively, in the *in vivo* P–388 screen. In the same P–388 screen, kaempferol 3-*O*-β-D-glucopyranoside (**3**) exhibited a T/C=122 and 130% when tested at 12.5 mg/kg. The good antileukemic activity (T/C=174% at 12.5 mg/kg) demonstrated by **2** is noteworthy, as the cytotoxic flavonoids seldom show significant *in vivo* activity against the P–388 lymphocytic leukemic growth in mice (19). It is also interesting to note that both **2** and **3** possess the same 7, 5 and 4' tri-hydroxylated pattern.

(+)-Nortrachelogenin (i.e. wikstromol) (**4**)[5] revealed activities of T/C=122, 130 and 130% at doses of 4, 8 and 16 mg/kg, respectively, when tested against *in vivo* P–388 lymphocytic leukemia growth in $BDF_1$ mice. The reported data for **4** in the same *in vivo* screen were T/C=130, 141, 137, 146 and 154% at doses of 1, 2, 4, 10 and 16 mg/kg in $CDF_1$ mice (4).[6]

## EXPERIMENTAL

EXTRACTION OF *W. indica* C. A. Mey.—The *W. indica* (Thymelaeaceae) used was from a collection made in August, 1978, at Mt. Kuan-Ying, Kaohsiung Shen, Taiwan.[7] The ground, air-dried, stems of this plant (4.55 kg) were exhaustively extracted with methanol, yielding, after removal of the solvent, a thick black tar. This was added to a mixture of methanol-water (1:1), 3 liters) and was then extracted with hexane and chloroform. Guided by both *in vivo* Ehrlich ascites (7) and P–388 (8) assays, the chloroform (46 g) and the residual methanol (486 g) extracts, which were found to be equally potent in both assays (e.g. >85% inhibition in the Ehrlich ascites assay as well as the T/C=160% and 156% at 25 mg/kg, respectively, in the P–388 assay), were combined. Upon evaporation of the solvent, a dark brown tar (522 g) was obtained.

ISOLATION OF DAPHNORETIN (**1**), TRICIN (5,7,4'-TRIHYDROXY-3',5'-DIMETHOXYFLAVONE) (**2**) AND KAEMPFEROL-3-*O*-β-D-GLUCOPYRANOSIDE (**3**).—The above-mentioned crude tar was chromatographed on silica gel (2 kg) and eluted with chloroform (9 liters), chloroform-ethyl acetate (9:1, 4 liters; 3:1, 6 liters; and 1:1, 11 liters), ethyl acetate (9 liters), ethyl acetate-methanol (9:1, 8 liters; 3:1, 7 liters; and 1:1, 3 liters), and methanol (3 liters).

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[4]Product of Sigma Chemical Company, St. Louis, MO.

[5]The name "wikstromol" (**4**) was first used by Tandon and Rastogi (17) for the lignan isolated from *Wikstroemia viridiflora* and was later used by Torrance, Hoffmann and Cole (4) for the antitumor principal isolated from *W. foetida* var. *oahuensis* and *W. uva-ursi*. Wikstromol {$[\alpha]_D+72°$ (C=0.37 in $CHCl_3$ (17) or $[\alpha]^{25}_D+41°$ (c=0.93 in $CHCl_3$ (4)} is actually the (+)-enantiomer of (−)-nortrachelogenin (**5**), isolated from *Trachelospermum asiaticum* var. *intermedium* by Nishibe and co-workers (18). Since (−)-nortrachelogenin {$[\alpha]^{17}_D-16.8°$ (c=0.178 in EtOH (18)} possesses a 8(S)8'(S) configuration as reported by Nishike (18), its (+)-enantiomer, (+)-nortrachelogenin (i.e. wikstromol) (**4**), would bear a 8(R)8'(R) configuration. Thus, the 8(S)8'(S) configuration shown for the formula of both (+)-nortrachelogenin {$[\alpha]_D+15.4$ (c=0.52 in $CHCl_3$ (3)} by Kato *et al.* (3) and wikstromol by Torrance *et al.* (4) were incorrect and all have to be inverted as depicted in **4** as was also noted by Miller (21).

[6]Dr. Matthew Suffness, National Cancer Institute, Bethesda, Md., personal communication, September 18, 1979.

[7]Collected and identified by H. C. Huang. A voucher specimen is available for inspection at the Herbarium of the School of Pharmacy, Kaohsiung Medical College, Kaohsiung, Taiwan, Republic of China.

DAPHNORETIN (**1**).—The active substance (**1**) obtained from elution with 6 liters of chloroform-methyl acetate (3:1) had mp 246–247° (tetrahydrofuran-methanol, 1:3; pale yellow needles; 1.2 g, 0.03% yield). Compound **1** exhibited uv[8] λ max (MeOH) nm (log ε) at 228 (shoulder, 4.19) 264 (3.95), 325 (4.23) and 342 (4.27) due to the presence of coumarin nucleus; high resolution ms, *m/z* (%) 352.0587 ($M^+$, 100) $C_{19}H_{12}O_7$ requires 352.0582), 324.0631 (M–CO, 2.5) ($C_{18}H_{12}O_6$ requires 324.0632), 179.0342 (16.7) ($C_9H_7O_4$ requires 179.0344), 146.0364 (2.6) ($C_9H_6O_2$ requires 146.0367) 337.0347 (M-Me, 2.4) ($C_{18}H_9O_7$ requires 337.0347), 309.0397 (M-Me-CO, 6.8) ($C_{17}H_9O_6$ requires 309.0397), and 281.0454 (M-ME-CO-CO, 1.2) $C_{16}H_9O_5$ requires 281.0449); and $^{13}$C-nmr (DMSO-$d_6$ δ, 160.0 (s) (C–2), 159.7 (s) (C–2'), 157.0 (s) (C–7'), 155.0 (s) (C–8'a), 150.4 (s), 147.5 (s), 145.7 (s), 144.1 (d) (C–4'), 135.7 (s), 130.9 (d), 130.0 (d) (C–5'), 114.4 (s), 113.9 (d), 113.5 (d) (C–3'), 110.2 (s), 109.4 (d), 104.0 (d), 102.8 (d) and 56.1 (s) (OMe).

The identity of **1** with an authentic sample of daphnoretin, isolated from *Daphne mezereum* (11), was established by tlc, ir, and nmr spectroscopic comparison and mixed melting point determination.

TRICIN (**2**) AND ITS ACETATE (**2a**).—The product obtained from fractions eluted by chloroform-ethyl acetate (1:1, 11 liters) was recrystallized from acetone; the resulting yellow needles (150 mg) had a mp of 276–279°; ms *m/z* 330.0732 ($C_{17}H_{14}O_7$ requires 330.0738), 315 [M–15 (M–Me)], 301 [M–29 (M–HCO)], 287 [M–43 (M–MeCO)], 181, 178, 163, 153 and 151[9]; ir: ν max 3580, 3520, ca. 3300 (OH), 1650 (hydrogen bonded α,β-unsat. CO), 1610, 1570, 1555, 1500 (aromatic) $cm^{-1}$; and uv λ max (MeOH) nm (log ε) at 268 (4.09) and 348 (4.19), +$AlCl_3$ at 255 (sh), 276, 305, 360 (sh) and 390. Lit. 17 reported uv λ max (MeOH) nm at 268 and 348, +$AlCl_3$ at 254 (sh), 276, 300 (sh), 359 and 387 (sh) for tricin. This compound was identical (mmp, ir, nmr and co-tlc) with an authentic sample of tricin (12). Acetylation of **3** with acetic anhydride in pyridine under reflux afforded a triacetate (3a): mp 249–250° (chloroform-methanol) [Lit. (19) reported mp 251–254°C (alcohol-acetic acid) for 5,7,4'-triacetoxy-3',5'-dimethoxy-flavone (i.e. triacetyl tricin)]; ms *m/z* 456.1052 ($C_{23}H_{20}O_{10}$ requires for 456.1055); and pmr ($CDCl_3$) δ 3.94 (6H, s, two $OCH_3$), 2.44 (3H, s), 2.36 (3H, s) and 2.35 (3H, s) (three $OCOCH_3$), 6.63 (1H, s, H–3), 7.09 (2H, s, H–2' and H–6'), 6.87 (1H, d, $J$=2.5 Hz, H–6) and 7.40 (1H, d, $J$=2.5 Hz, H–8).

KAEMPFEROL-3-*O*-β-D-GLUCOPYRANOSIDE (**3**), ITS ACETATE (3a) AND HYDROLYSATE (3b).—The active ethyl acetate (9 liters) and ethyl acetate-methanol (9:1, 8 liters) eluates were combined (30 g) and rechromatographed on silica gel (1.5 kg). Elution of the column with chloroform-methanol-water (50:12:3) yielded the active yellow needles (**3**, 123 mg). Compound **3** had mp 235–237° (acetone); $[\alpha]^{25}_D$ −60° (c=0.0012, MeOH); and had ir, pmr and mass spectra identical with those of an authentic sample of kaempferol-3-*O*-β-D-glucopyranoside. Compound **3** formed, upon treatment with acetic anhydride-pyridine, a hepta acetate (3a): mp 214–215° (chloroform-methanol); *m/z* 742.1749 ($C_{35}H_{34}O_{18}$ requires 742.1742); and pmr ($CDCl_3$ δ 1.94 (3H, s, glucose 6-OAc), 2.02, 2.04 and 2.14 (3H, s, each, glucose 2–, 3–, and 4– OAc). 2.37 (6H, s) and 2.48 (3H, s) (flavone OAc). Acid hydrolysis of **2** with 1:1 methanol-10% hydrochloric acid yielded an aglycon (3b, yellow needles): mp 274–276° (dec) (methanol); *m/z* 286.0473 ($C_{15}H_{10}O_6$ requires 286.0476). This aglycon was identical (superimposable tlc, ir, nmr spectra and undepressed mixed mp) with an authentic sample of kaempferol (from Sigma Chemical Co.).

ISOLATION OF (+)-NORTRACHELOGENIN (**4**).—In a separate experiment, the ethanolic extract of the same plant (4 kg) was extracted successively with hexane (1 liter) and chloroform (2 liters) to yield hexane-soluble and chloroform-soluble fractions. The latter fraction was chromatographed on hyflosupercel (350 g) and eluted with benzene and chloroform (4 liters). The benzene eluate was further extracted successively with 10% sodium bicarbonate (500 ml) and 1% sodium hydroxide (500 ml). Chromatography of the 1% sodium hydroxide-soluble fraction on silica gel gave compound **4** [30 mg (0.0015% yield); colorless powder; ms *m/z* 374 ($M^+$)] whose specific rotations, uv, ir, pmr and mass spectra were identical with those of the authentic (+)-nortrachelogenin (3).

The chloroform eluate (22 g) was rechromatographed on Florisil (100 g) and eluted with ethyl acetate to afford 250 mg (0.0125% yield) of yellow crystalline daphnoretin (**1**).

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[8]Lit. 4 reported uv λ max (MeOH) nm (log ε) at 228 (1.18, 265 (0.86), 325 (1.28) and 343 (1.31). Lit. 11 reported uv λ max (MeOH) nm (log ε) at 228 (4.18, shoulder), 265 (3.86), 325 (4.28) and 343 (4.31).

[9]*M/z* 181, 178, 163, 153 and 151 were fragment ions resulting from a retro Diels-Alder reaction of **3**, and were in accord with those observed by Mabry and associates (20) for the methylated flavonoids.

Whiteknights, Reading, for a sample of tricin; Professor A. Kato, Kobe Women's College of Pharmacy, for a sample of (+)-nortrachelogenin; Professors Jack R. Cole and Joseph J. Hoffman, College of Pharmacy, University of Arizona, for a sample of wikstromol; Dr. D. L. Harris, Department of Chemistry, University of North Carolina, for XL-100 pmr spectra, and Dr. David Rosenthal and Mr. Fred Williams of the Research Triangle Center for Mass Spectrometry for mass spectral data.

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