

## Antineoplastic Agents. 573. Isolation and Structure of Papilistatin from the Papilionid Butterfly *Byasa polyeuctes termessa*<sup>†</sup>

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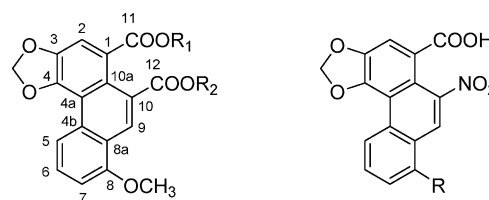
Bioassay-guided separation of an extract of the wings from a Taiwan butterfly, *Byasa polyeuctes termessa*, allowed isolation of a new cancer cell growth inhibitor designated papilistatin (**1a**). The structure was determined by analysis of 1D and 2D NMR spectra and by HRMS. Against a panel of six human and the murine P388 leukemia cancer cell lines, papilistatin exhibited cancer cell growth inhibition with GI<sub>50</sub>'s of 0.093–3.5 μg/mL. Papilistatin was also found to have antibacterial activity.

Insects are now believed to number some four to six million species,<sup>2</sup> with butterflies and moths (order Lepidoptera) comprising over 100 000 species.<sup>3</sup> Such an enormous reservoir of as yet unexplored compounds that could include biologically active structures is most compelling. Recent examples that provide some insight into the scope of this potential reservoir include the pheromones of the giant white butterfly *Idea leuconoe*,<sup>4a</sup> spiroacetals from a parasitoid wasp (order Hymenoptera),<sup>4b</sup> the defensive spray of a walkingstick insect (order Phasmatodea),<sup>4c</sup> and the anticancer drug pancratistatin from a grasshopper (order Saltatoria).<sup>4d</sup> The medically important toxins of certain Brazilian centipede (class Chilopoda) venoms present another dimension.<sup>5</sup> Investigations of other Arthropoda classes such as the arachnids, comprising nearly 40 000 species, are leading to discoveries of new and potentially useful constituents.<sup>6</sup> Illustrative are the p53-MDM2/MDMX inhibitor, derived from a toxin of an Asian scorpion,<sup>6a</sup> and the sulfated nucleoside constituents of a spider venom.<sup>6b</sup>

In 1965–1966, we began to pioneer the first systematic investigation of arthropods for antineoplastic components and summarized the initial promising results in 1968.<sup>3</sup> Our most recent advance in that area was the isolation from a Texas grasshopper of pancratistatin,<sup>4d</sup> a preclinical anticancer drug that had in the meantime been isolated by us from an African plant. Another class Insecta metabolite investigation that was delayed until recently pertains to the papilionid butterfly *Byasa polyeuctes termessa* (*Atrophaneura polyeuctes termessus* Fruhstorfer), which was collected in Taiwan in 1967. We now report the isolation and structure of a new cancer cell growth inhibitor from the butterfly, designated papilistatin (**1a**).

To our knowledge, no prior chemical studies of *B. polyeuctes* have been reported, nor of the genus, except that one member, *Byasa alcinous* (*Atrophaneura alcinous* Klug), was investigated for papilochromes<sup>7</sup> and butyric acids<sup>8</sup> with negative results. In addition, it was reported that *B. alcinous* is attracted to *Aristolochia debilis*,<sup>9</sup> one of many Aristolochiaceae species that are used as herbal folk remedies. The active compounds that have been isolated from these plants<sup>10,11</sup> include the aristolochic acids (AA), of which aristolochic acid I (**2a**) and aristolochic acid II (**2b**) are the major components; also in the active extracts are aristolactams and terpenoids. Kupchan and Dorskotch<sup>10a</sup> reported in 1962 that a sample of **2a** from *A. indica* was active in the U.S. National Cancer Institute's in vivo adenocarcinoma 755 evaluation system, and two years later its first phase

I human cancer clinical trial was reported.<sup>12</sup> Subsequently, **2a** (or the AA mixture) was linked to the development of tumors in mice and rats, and AA was associated with the carcinogenicity and nephrotoxicity of certain Chinese and Balkan herbs.<sup>13a–d</sup> The evidence now indicates that **2a** and **2b** form DNA adducts that are genotoxic mutagens, following metabolic activation via reduction of the nitro group.<sup>13a–c</sup>



**1a**, R<sub>1</sub> = R<sub>2</sub> = H

**1b**, R<sub>1</sub>, R<sub>2</sub> = H, C<sub>5</sub>H<sub>6</sub>N

**2a**, R = OCH<sub>3</sub>

**2b**, R = H

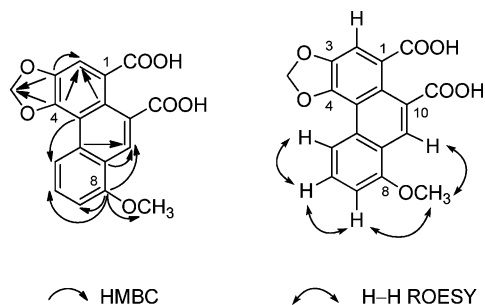
### Results and Discussion

A 1967 collection of *B. polyeuctes termessa* was dissected, and the anticancer activity was located in the wings. In an assay at the U.S. National Cancer Institute using random-bred albino rats bearing the Walker 256 carcinoma (subcutaneous), a 95% ethanol extract of the wings led to a 68–78% (at 400 mg/kg) reduction in tumor growth by day 10 and was judged a confirmed active. A solution of the ethanol extract (4.8 g) in CH<sub>3</sub>OH–CH<sub>2</sub>Cl<sub>2</sub> (9:1) was filtered to collect the solid phase (0.11 g). The solution was concentrated to dryness and taken up in CH<sub>3</sub>OH. A yellow, amorphous powder (48 mg) that separated was removed, and the remaining solution was fractionated on a Sephadex LH-20 column, with CH<sub>3</sub>OH as eluent. The P388-active fractions were further separated by successive column chromatographic procedures using Sephadex LH-20. Final separation and purification was achieved by repeated reversed-phase high-performance liquid chromatography (CH<sub>3</sub>CN–H<sub>2</sub>O or CH<sub>3</sub>OH–H<sub>2</sub>O) to afford papilistatin (**1a**, 15 mg) as a yellow powder.

The structure of **1a** was determined by detailed spectroscopic analyses. The IR spectrum showed hydroxyl group (3400 cm<sup>-1</sup>) and aromatic ring (1590, 1395 cm<sup>-1</sup>) absorptions. Resonances in the <sup>1</sup>H NMR spectrum were indicative of an OCH<sub>3</sub> group (δ 3.89), a 1,2,3-substituted aromatic ring (doublets at δ 7.13 and 8.73 and a triplet at δ 7.75), and another two aromatic protons (singlets at δ 8.21 and 8.96). In the HMBC NMR spectrum, the carbon signal at δ 156.9 (s) showed long-range correlations with protons at δ 3.89 (3H, s), 7.13 (1 H, d), 7.75 (1 H, dd), and 8.96 (1H, s), supporting the placement of a methoxy group at C-8, which was confirmed

<sup>†</sup> Dedicated to the memory of Professor Georgy B. Elyakov (1929–2005), a pioneering expert in the chemistry of naturally occurring substances, especially from marine organisms.

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**Figure 1.** Key HMBC and ROE correlations for papilistatin (**1a**).

by the ROESY correlations between the methyl protons at  $\delta$  3.89 and the two protons at  $\delta$  7.13 and 8.96 (Figure 1). The HMBC correlation between the two protons at  $\delta$  6.34 and the carbons at  $\delta$  146.5 (C-3) and 146.3 (C-4) supported the placement of the methylene group. The structures of **1a** and **2a** are closely related, suggesting the possibility that *B. polyeuctes* acquires **1a** by feeding on an *Aristolochia* species. Compounds **2a** and **2b** have been found in other southeast Asian swallowtail butterflies that feed on AA-containing *Aristolochia* species, and their assumed role in chemical defense is under investigation.<sup>14</sup> Presumably, the *Byasa* species concentrates papilistatin (**1a**) from such a plant source, possibly for a variety of defensive purposes, which should stimulate future biological studies.

When screened against a panel of six human cancer cell lines and the P388 lymphocytic leukemia, **1a** exhibited strong selective activity against the colon KM20L-2 and pancreas BXP-3 lines, with  $GI_{50}$  values of 0.096 and 0.093  $\mu\text{g/mL}$ , respectively (see Experimental Section). Against a panel of bacteria and fungi, **1a** had primarily anti-Gram-positive antibacterial activity (see Experimental Section), inhibiting growth of *Streptococcus pneumoniae* (minimum inhibitory concentration [MIC] 32  $\mu\text{g/mL}$ ), *Micrococcus luteus* (MIC 16–32  $\mu\text{g/mL}$ ), *Staphylococcus aureus* (MIC 64  $\mu\text{g/mL}$ ), and *Enterococcus faecalis* (MIC 16–32  $\mu\text{g/mL}$ ). Compound **1a** also inhibited growth of the pathogenic Gram-negative bacterium *Neisseria gonorrhoeae* (MIC 8–16  $\mu\text{g/mL}$ ).

## Experimental Section

**General Experimental Procedures.** Solvents used for column chromatography were distilled. Sephadex LH-20 (particle size 25–100  $\mu\text{m}$ ) used in gel permeation and partition column chromatographic separations was obtained from Pharmacia Fine Chemicals AB, Uppsala, Sweden. The TLC plates were viewed under shortwave UV light and then developed by 10%  $\text{H}_2\text{SO}_4$  spray reagent followed by heating at approximately 150  $^\circ\text{C}$ . For HPLC separations, a Phenomenex Luna (particle size 5  $\mu\text{m}$ , 250 mm  $\times$  10.0 mm)  $\text{C}_{18}$  column and a Phenomenex Luna (particle size 5  $\mu\text{m}$ , 250 mm  $\times$  4.6 mm)  $\text{C}_{18}$  column were used in reversed-phase mode with a Waters Delta (model 600E) solvent metering pump and a Waters 2487 Dual  $\lambda$  absorbance detector. IR spectra were recorded using an Avatar 360 FT-IR instrument with the sample prepared in a  $\text{CH}_2\text{Cl}_2$  film. The UV spectrum was recorded with a Perkin-Elmer 336. The NMR experiments were conducted using a Varian Unity Inova 500 spectrometer operating at 500 and 120 MHz for  $^1\text{H}$  and  $^{13}\text{C}$  NMR, respectively.

***Byasa polyeuctes termessa*.** The papilionid butterfly was obtained in various areas of Taiwan in 1967 by collectors employed by the Butterfly Company of New York, New York, under the supervision of professional entomologists, and supplied to our Institute. Such collections usually involved several thousand specimens of each species, and voucher specimens of this *Byasa* species have been maintained in the ASU Cancer Research Institute.

**Isolation of Papilistatin (**1a**).** Isolation of an active cancer cell growth inhibitor from the *Byasa* species was conveniently realized as follows. The butterfly was first dissected into a cross section of anatomical parts, principally the heads, abdomens, thoraxes, and wings. At that time (1967–1969), the initial anticancer activity was detected in 95% ethanol extracts of the wings using the Walker 256 carcinoma

(subcutaneous) in random-bred albino rats under the auspices of the U.S. National Cancer Institute.<sup>3</sup> The extract was given intraperitoneally (in saline) on the first day of tumor transplant and repeated over each of five days, with growth of the tumor measured on day 10. The result was 68–78% (at 400 mg/kg) growth inhibition, a confirmed active level.

Prior to preparation of the ethanol extract, the wings (83 g) were given a pretreatment with petroleum ether and 1:1 ethanol– $\text{H}_2\text{O}$ <sup>3</sup> to yield petroleum ether (3.5 g), 50% ethanol (11.0 g), and 95% ethanol (4.8 g) extracts. By means of bioassay-guided separation using the murine P388 lymphocytic leukemia, the isolation of papilistatin (**1a**) proceeded as follows. The 95% ethanol extract (4.8 g) was taken up in 9:1  $\text{CH}_3\text{OH}$ – $\text{CH}_2\text{Cl}_2$ , and the less soluble material (0.11 g) was removed. Removal of solvent from the solution left a residue, which was dissolved in  $\text{CH}_3\text{OH}$  (48 mg of insoluble material was removed) and subjected to gel permeation chromatography on Sephadex LH-20. A total of eight cancer-inhibitory fractions (4.7 g altogether) were recovered. Two of these fractions were combined (1.2 g) and subjected to successive chromatographic separations on Sephadex LH-20 in  $\text{CH}_3\text{OH}$ – $\text{CH}_2\text{Cl}_2$  (2:3), hexane–2-propanol– $\text{CH}_3\text{OH}$  (8:1:1), and hexane– $\text{CH}_2\text{Cl}_2$ –acetone (4:3:3) as eluents. Final purification by employment of repeated HPLC in 5:95 to 4:1  $\text{CH}_3\text{CN}$ – $\text{H}_2\text{O}$  afforded papilistatin (**1a**) as a yellow, amorphous powder (15 mg): UV (EtOH)  $\lambda_{\text{max}}$  322, 285 nm; IR ( $\text{CH}_2\text{Cl}_2$ )  $\nu_{\text{max}}$  3400, 2960, 2880, 1731, 1590, 1395, 1350, 1275, 1050, 820, 780  $\text{cm}^{-1}$ ;  $^1\text{H}$  NMR ( $\text{C}_5\text{D}_5\text{N}$ , 500 MHz)  $\delta$  8.96 (1H, s, H-9), 8.73 (1H, d,  $J = 8.5$  Hz, H-5), 8.21 (1H, s, H-2), 7.75 (1H, dd,  $J = 8.5, 8.0$  Hz, H-6), 7.13 (1H, d,  $J = 8.0$  Hz, H-7), 6.34 (2H, s,  $\text{OCH}_2\text{O}$ ), 3.89 (3H, s,  $\text{OCH}_3$ );  $^{13}\text{C}$  NMR ( $\text{C}_5\text{D}_5\text{N}$ , 120 MHz)  $\delta$  169.8 (s, C-11), 156.9 (s, C-8), 147.3 (s, C-12), 146.5 (s, C-3), 146.3 (s, C-4), 131.2 (d, C-6), 131.0 (s, C-4b), 126.6 (s, C-1\*), 120.3 (d, C-9), 120.1 (s, C-4a), 119.4 (d, C-5), 118.6 (s, C-8a), 118.4 (s, C-10a), 113.1 (d, C-2), 108.4 (d, C-7), 103.0 (t,  $\text{OCH}_2\text{O}$ ), 97.5 (s, C-10\*), 56.0 (q,  $\text{OCH}_3$ ) (assignments marked with an asterisk are interchangeable); HRMS (APCI+)  $m/z$  324.0615 (calcd for  $\text{C}_{18}\text{H}_{12}\text{O}_6 + 2\text{H} - \text{H}_2\text{O}$ , 324.0634).

From a pyridine–hexane solution, yellow crystals of papilistatin pyridinium salt **1b** were obtained: mp 262.1  $^\circ\text{C}$  (dec).

**Cancer Cell Line Evaluations.** The  $GI_{50}$  ( $\mu\text{g/mL}$ ) results for papilistatin (**1a**) against six human cancer cell lines and the murine P388 lymphocytic leukemia are as follows: breast MCF-7 (3.5), CNS-28 (>1), colon KM20L-2 (0.096), lung NCI-H460 (>1), pancreatic BXP-3 (0.093), prostate DU-145 (>1), and P388 leukemia (3.4). These experiments were conducted as previously described.<sup>15</sup>

**Antimicrobial Susceptibility Testing.** Compound **1a** was screened against the bacteria *Stenotrophomonas maltophilia* ATCC 13637, *Micrococcus luteus* Presque Isle 456, *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922, *Enterobacter cloacae* ATCC 13047, *Enterococcus faecalis* ATCC 29212, *Streptococcus pneumoniae* ATCC 6303, and *Neisseria gonorrhoeae* ATCC 49226 and against the fungi *Candida albicans* ATCC 90028 and *Cryptococcus neoformans* ATCC 90112, according to established broth microdilution susceptibility assays.<sup>16,17</sup>

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**Supporting Information Available:** NMR spectra of compound **1a**. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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