A Cytotoxic Sesquiterpene Caffeate from the Liverwort *Bazzania novae-zelandiae*

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Extracts of the liverwort *Bazzania novae-zelandiae* were cytotoxic, with selective activity against human tumor cell lines. Bioactivity-directed isolation work showed that the new compound naviculyl caffeate (**1**) was the main cytotoxin. This structure was confirmed by synthesis from the co-occurring sesquiterpene naviculol (**2**).

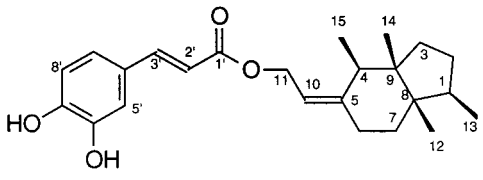
Esters of caffeic acid occur widely in plants and some have biological activities against human cancers and viruses. The di-caffeate of tartaric acid, chicoric acid, is active against the human immunodeficiency virus,[1] and 3-methylbut-2-enyl caffeate is active against herpes simplex virus.[2] 2-Phenylethyl caffeate, which occurs with 3-methylbut-2-enyl caffeate in propolis,[3] showed preferential cytotoxicity on tumor cells.[4] We now report the isolation and structure determination of a new cytotoxic sesquiterpene caffeate from *Bazzania novae-zelandiae* (Mitt.) Besch. & Massal. (family Lepidoziaceae).

B. novae-zelandiae is a robust liverwort that grows throughout New Zealand and the sub-Antarctic Islands and has also been recorded from South America.[5] The only report on the chemistry of *B. novae-zelandiae* describes three sesquiterpene hydrocarbons, β-chamigrene, β-bazzanene and β-barbatene as the main components by GC−MS.[6] *Bazzania* species have a worldwide distribution,[5] and a variety of natural products have been reported from at least 13 species.[7] The most relevant to this work are four sesquiterpene caffeates from *B. fauriana*, *B. japonica*, and *B. pompeana*.[8−10] Among the liverworts, sesquiterpene caffeates have only been found in *Bazzania*,[7] and we could not find reports of such compounds from other organisms. The only biological activity recorded for these compounds is inhibition of superoxide release.[10] The U.S. National Cancer Institute (NCI) found significant in vivo antitumor activity against P-388 leukemia for an extract of *B. trilobata*, but the compound responsible was not isolated.[11]

Two collections of *B. novae-zelandiae*, included in our screening of New Zealand plants for bioactive natural products,[12] gave extracts with cytotoxic activity against P-388 leukemia cells. Both extracts showed some selective cytotoxicity in the NCI's panel of human tumor cell lines.[13] A bulk extract was subjected to reversed-phase flash chromatography, which concentrated the cytotoxic activity in fractions with ^{1}H NMR signals characteristic of (*E*)-caffeate groups (16 Hz doublets at 6.26 and 7.56 ppm).[10] Si gel flash chromatography further concentrated the cytotoxic activity, but attempts at final purification by preparative reversed-phase HPLC gave samples whose ^{1}H NMR spectra contained 13 Hz doublets (5.80 and 6.75 ppm) not seen in preceding stages of the fractionation or in the crude extract. These were assigned as (*Z*)-caffeate signals, presumably formed from the natural (*E*)-caffeate by exposure to UV light in aqueous solution.[14,15] Preparative reversed-phase HPLC, with fractions collected and concentrated in low daylight (no fluorescent lights), was successful in giving a pure sample of (*E*)-caffeate (**1**).

HRFABMS supported the molecular formula $C_{24}H_{32}O_4$, and the (*E*)-caffeate portion of the molecule was confirmed by UV (λ_{max} 326 nm) and ^{1}H and ^{13}C NMR (Table 1). The structure of the remainder of the molecule was solved by a combination of NMR experiments (Table 1). Only one further carbon−carbon double bond was present, so the $C_{15}H_{25}$ portion had to be bicyclic. A three-bond ^{1}H−^{13}C correlation (HMBC) between the ester carbonyl and two methylene protons at 4.71 ppm showed that the parent C_{15} alcohol was primary. COSY and HMBC correlations showed that this methylene was linked to a trisubstituted double bond. Further COSY and HMBC correlations (Table 1) allowed us to propose structure **1** for the compound from *B. novae-zelandiae*. NOE interactions between the H-11 protons and H-5 and between H-10 and one H-6 showed that the 5−10 double bond was *Z*. The methyl signals were not sufficiently resolved for NOE assignment of their relative stereochemistry.

A search of the Chemical Abstracts Registry file for the $C_{15}H_{25}O$ portion of structure **1** led to naviculol (**2**), which has been found in two liverworts, *Porella navicularis*[16] and *Frullanoides densifolia*.[17] Our NMR data for **1** (Table 1) compared well with those reported for **2**, allowing for the acylation at C-11 and reversing the previous assignments for C-2 and C-3.[16]



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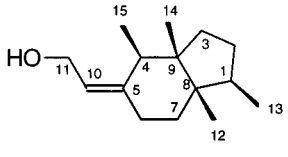

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We found naviculol (**2**) in *B. novae-zelandiae* extracts, in less polar fractions than those containing **1**. The relative and absolute stereochemistry of naviculol (**2**) has been

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Table 1. NMR Data for Naviculyl Caffeate (**1**)[a]

position	^{13}C	^{1}H[b]	COSY	HMBC[c]
1	43.9	1.75, m	0.91	0.91, 1.27
2β	30.3	1.27, m	1.88	0.91, 1.14, 1.72
2α		1.88, dtd, 13, 9, 2	1.27, 1.72	
3β	38.1	1.12, ddd, 13, 9, 2	1.27, 1.72	0.94, 1.27, 1.88, 2.50
3α		1.72, m	1.12, 1.88	
4	40.6	2.50, q, 7	1.10	0.94, 1.10, 1.72, 2.01, 5.40
5	150.3			1.10,1.36, 1.46, 2.01, 2.35, 2.50, 4.71, 5.40
6α	29.9	2.01, dt, 14, 5	2.36, 1.46	1.36, 1.46, 2.50, 4.71, 5.40
6β		2.36, br ddd, 13, 11, 5	2.01, 1.46	
7β	39.3	1.36, dt, 14, 5	1.46, 1.27	0.94, 1.75, 2.01, 2.35
7α		1.46, ddd, 13, 11, 5	2.35, 2.01, 1.36	
8	44.2			0.91, 0.93, 1.14,1.36, 1.46, 1.75, 1.88, 2.01, 2.50[d]
9	47.9			0.93, 0.94, 1.10, 1.14, 1.36, 1.46, 1.72, 1.88, 2.50[d]
10	116.0	5.40, t, 7	4.71	2.01, 2.36, 2.50, 4.71, 7.57[e]
11	60.9	4.70, ddd, 12, 7, 1	5.40	5.40
11		4.72, dd, 12, 7	5.40	
12	19.3	0.93, s		0.93, 1.27, 1.36, 1.46, 1.75, 1.88
13	19.2	0.91, d, 7	1.75	0.93, 1.27, 1.36, 1.46, 1.75, 1.88
14	21.8	0.94, s		none seen
15	16.4	1.10, d, 7	2.50	2.50, 5.40
1′	168.0			4.71, 6.25, 7.57
2′	115.5	6.25, d, 16	7.57	6.98[e], 7.57
3′	145.0	7.57, d, 16	6.25	6.25, 6.98, 7.08
4′	127.5			6.25, 6.86, 7.08, 7.57
5′	114.3	7.08, d, 2	6.98	6.86[e], 6.98, 7.57
6′	143.9			6.86, 7.08
7′	146.5			6.86, 6.98, 7.08
8′	115.4	6.86, d, 8	6.98	none seen
9′	122.4	6.98, dd, 8, 2	6.86, 7.08	6.86, 7.08, 7.57
6′-OH		6.12, br s		
7′-OH		6.30, br s		

[a] In $CDCl_3$, ^{1}H at 500 MHz, ^{13}C at 125 MHz. [b] Shift, multiplicity, couplings in Hz; positions of overlapped signals from HMBC. [c] ^{1}H signals correlated to ^{13}C. [d] Correlations from overlapping signals. [e] Long-range correlation.

Table 2. Cytotoxic Activities of Caffeates

sample	P-388 GI_{50} (μg/mL)[a]
naviculyl caffeate (**1**)[b]	1.1 (1.2, 1.1)
naviculyl caffeate (**1**)[c]	0.8 (0.91, 0.62)
bazzanenyl caffeate	0.9 (0.94, 0.87)
3-methylbut-2-enyl caffeate	6.9 (7.4, 6.4)
naviculol (**2**)	> 25
caffeic acid	> 25
chicoric acid	> 25

[a] Mean concentration for 50% growth inhibition (replicate values). [b] Natural. [c] Semisynthetic.

established by Toyota et al.[16] Naviculol from *B. novae-zelandiae* had the same absolute stereochemistry, as its optical rotation ($[\alpha]_D$ +44°) was very similar to that reported previously (+48.5°). We synthesized naviculyl caffeate (**1**) from naviculol (**2**) in order to confirm the structure, to establish the absolute stereochemistry of **1**, and to give enough material for further testing at the NCI. Dicyclohexylcarbodiimide (DCC) was used as a chemical dehydrating agent for a direct reaction between naviculol (**2**) and caffeic acid. The semisynthetic sample of naviculyl caffeate was identical with the natural product **1** by optical rotation and by ^{1}H and ^{13}C NMR spectroscopy.

Naviculyl caffeate (**1**) isolated from *B. novae-zelandiae* had moderate activity against P-388 murine leukemia, and assays on a semisynthetic sample of **1** confirmed this activity (Table 2). Bazzanenyl caffeate, from *B. fauriana*,[9] showed P-388 activity similar to that of **1**. 3-Methylbut-2-enyl caffeate, synthesized in a model reaction for the preparation of **1**, showed somewhat lower P-388 activity. Cytotoxic activities of 3-methylbut-2-enyl caffeate and bazzanenyl caffeate have apparently not been reported previously. Naviculol (**2**), caffeic acid, and chicoric acid, isolated from the medicinal herb *Echinacea purpurea*,[18] did not show any P-388 activity at the levels tested (Table 2).

Semisynthetic naviculyl caffeate (**1**) had a mean GI_{50} (50% growth inhibition) of 2.5 μg/mL tested against the NCI's panel of 60 human tumor cell lines.[13] The most susceptible lines were a leukemia (GI_{50} 0.4 μg/mL, CCRF−CEM) and a nonsmall-cell lung cancer (GI_{50} 0.5 μg/mL, NCI−H522).

A literature search for cytotoxic or antitumor activity of caffeates showed that these compounds do not generally show such bioactivity. Grunberger et al. have tried to explain the preferential cytotoxicity of 2-phenylethyl caffeate on tumor cells.[4] Because neither 2-phenylethanol nor caffeic acid showed cytotoxic effects, these researchers speculated that esterification of caffeic acid with a lipophilic alcohol facilitated transport into cells. In the cells, hydrolysis might release caffeic acid to give cytotoxic effects. A similar mechanism could explain the cytotoxicities of naviculyl caffeate (**1**), bazzanenyl caffeate, and 3-methylbut-2-enyl caffeate and the inactivity of the hydrophilic chicoric acid (Table 2).

Experimental Section

General Experimental Procedures. All solvents were distilled before use and were removed by rotary evaporation at temperatures up to 35 °C. Octadecyl-functionalized Si gel (C_{18}) was used for reversed-phase flash chromatography, and Davisil, 35−70 μm, 150 Å was used for Si gel flash chromatography. TLC was carried out using Merck DC-plastikfolien Kieselgel 60 F_{254}, first visualized with a UV lamp, and then by dipping in a vanillin solution (1% vanillin, 1% H_2SO_4 in EtOH) and heating. Optical rotations were measured on a Perkin-Elmer 241 polarimeter. MS, UV, and IR spectra were recorded on Kratos MS-80, Shimadzu UV 240, and Perkin-Elmer 1600 FTIR instruments, respectively. NMR spectra, at 25 °C, were recorded at 500 or 300 MHz for ^{1}H and 125 or 75 MHz for ^{13}C on Varian INOVA-500 or VXR-300 spectrometers. Chemical shifts are given in parts per million (ppm) on the δ

scale referenced to the solvent peak C*H*Cl_3 at 7.25 and *C*DCl_3 at 77.0. For the P-388 assay a two-fold dilution series of the sample was incubated for 72 h with murine leukemia cells (ATCC CCL 46 P-388D1). The concentration of the sample required to inhibit cell growth to 50% of the growth of a solvent control was determined using the absorbance obtained upon staining with MTT tetrazolium. As a positive control for this assay, mitomycin C, at a concentration of 0.06 μg/mL, inhibited the growth of P-388 cells by 43−75%.

Collection and Extraction. *B. novae-zelandiae* was collected from Port Adventure, Stewart Island, New Zealand, in January 1994 [PERU code 940127−04, University of Otago Herbarium (OTA) specimen 046765] and from the Charleston area, West Coast, South Island, in January 1997 (970129-11). Initial screening was carried out using extracts produced by shaking air-dried (30 °C), ground material (5.0 g) overnight in EtOH (50 mL).

Isolation of Naviculyl Caffeate (1). A bulk extract of collection 970129−11 (67 g) was prepared by blending with EtOH (1 × 350 mL, 3 × 150 mL) then $CHCl_3$ (2 × 150 mL). The solvent was removed from the combined, filtered extracts to give a green gum (1.92 g, P-388 GI_{50} 5.4 μg/mL). The crude extract was subjected to reversed-phase flash chromatography (1.92 g precoated on 2 g C_{18}, loaded on a 20-g C_{18} column), developed in 15-mL steps from H_2O through MeCN to $CHCl_3$. Fractions eluted with H_2O−MeCN (1:9), MeCN, and $CHCl_3$ had GI_{50}s < 10 μg/mL).

The least polar reversed-phase fraction (1.01 g, eluted with 1:1 $CHCl_3$−MeCN, P-388 GI_{50} 8.2 μg/mL) was subjected to flash chromatography on Si gel (12 g), developed in steps from cyclohexane to EtOAc. Fractions eluted with 6:1, 5:1, and 4:1 cyclohexane−EtOAc, which showed a UV-active spot on TLC (2:1 cyclohexane−EtOAc) at R_f 0.3, were combined (88 mg).

The main component in this sample was purified by reversed-phase HPLC (Merck Lichrospher 100 RP_{18}, 250 × 10 mm, mobile phase 5 mL/min 15% H_2O−85% MeOH, detection at 206 nm). Compound **1** (3 mg), with retention time 25.5 min, was recovered by adding H_2O (35 mL) and CH_2Cl_2 (35 mL) to combined fractions (35 mL), separating the organic layer, and removing the solvents at room temperature with no fluorescent lighting in a flask painted black.

Naviculyl caffeate (1): colorless oily gum; $[\alpha]^{28}_D$ +28°, $[\alpha]^{28}_{577\ nm}$ +12°, $[\alpha]^{28}_{546\ nm}$ +4°, $[\alpha]^{28}_{435\ nm}$ −68°, $[\alpha]^{28}_{405\ nm}$ −76° (*c* 0.2, MeOH); UV (MeOH) λ_{max} (log ϵ) 244 (3.66), 300 (shoulder), 326 (3.88) nm; IR (film) ν_{max} 3500−3100, 2927, 1678, 1600, 1443, 1276, 1150 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; FABMS *m*/*z* 384.2326 (20, calcd for $C_{24}H_{32}O_4$, 384.2301), 205 (100); P-388 GI_{50}, see Table 2.

Isolation of naviculol (2). A bulk extract of collection 940127-04 (25 g) was prepared by Soxhlet extraction with $CHCl_3$ (200 mL, 20 h). The solvent was removed to give a green gum (1.14 g). The crude extract was subjected to Si gel flash chromatography (1.13 g precoated on 2.2 g, loaded on a 20-g column), developed in steps from cyclohexane to EtOAc. A fraction eluted with 8:2 cyclohexane−EtOAc (90 mg) was subjected to reversed-phase HPLC (as above). Compound **2** (14 mg) had a retention time of 13.2 min.

Naviculol (2): colorless gum; $[\alpha]^{20}_D$ +44° (*c* 0.2, $CHCl_3$); IR (film) ν_{max} 3320 (broad), 2927, 1450, 1009 cm^{-1}; ^1H and ^{13}C NMR as previously reported.[16]

Synthesis of 3-Methylbut-2-enyl Caffeate. A solution of caffeic acid (Aldrich, 57 mg, 0.32 mmol), 3-methylbut-2-enol (Merck, 37 mg, 0.42 mmol), and DCC (Aldrich, 66 mg, 0.32 mmol) in dry THF (2 mL) was stood at room temperature in the dark for 18 h. The solution was concentrated to give an orange oil. Column chromatography over Si gel eluting with 0−100% EtOAc in CH_2Cl_2 gave the crude ester (71 mg). A CH_2Cl_2 solution of this was stirred with polyvinylpyrrolidine (PVP) for 15 min, then column chromatography over PVP eluting with CH_2Cl_2−EtOAc−MeOH gave the pure ester as a white crystalline solid (33 mg, 41%): registry number 117614-99-0; mp (EtOAc−Hexane) 121 °C.

Synthesis of Naviculyl Caffeate (1). A solution of caffeic acid (20 mg, 0.11 mmol), naviculol (24 mg, 0.11 mmol), and DCC (25 mg, 0.12 mmol) in dry THF (2 mL) was held at room temperature in the dark for 18 h. The solution was concentrated to give an orange oil. Column chromatography over Si gel, eluting with 0−100% EtOAc in CH_2Cl_2, gave recovered naviculol (9 mg, 37%), followed by the pure ester **1** (11 mg, 28%): colorless gum; $[\alpha]^{28}_D$ +29°, $[\alpha]^{28}_{577\ nm}$ +16°, $[\alpha]^{28}_{546\ nm}$ +11°, $[\alpha]^{28}_{435\ nm}$ −147°, $[\alpha]^{28}_{405\ nm}$ −180° (*c* 0.5, MeOH); ^1H and ^{13}C NMR matched the natural product, see Table 1; P-388 GI_{50}, see Table 2.

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