New *Cephalotaxus* Alkaloids from *Cephalotaxus harringtonia* var. *drupacea*

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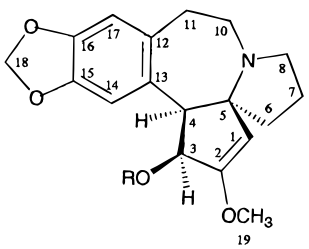
The cytotoxic alkaloids nordeoxyharringtonine (**2**), homodeoxyharringtonine (**3**), and bishomodeoxyharringtonine (**4**) were isolated from the leaves and stems of *Cephalotaxus harringtonia* var. *drupacea*. Their structures were established on the basis of spectral data and chemical evidence.

The genus *Cephalotaxus* (Cephalotaxaceae) has long been known to contain antileukemic alkaloids such as harringtonine, homoharringtonine, isoharringtonine and deoxyharringtonine (**1**).[1−3] A recent investigation in our search for novel antileukemic compounds from native Japanese plants has resulted in the isolation of three congeners of **1**, nordeoxyharringtonine (**2**), homodeoxyharringtonine (**3**), and bishomodeoxyharringtonine (**4**) from *C. harringtonia* var. *drupacea* (Sieb. & Zucc.) Koidzumi (Cephalotaxaceae). Compounds **2** and **4** proved to be novel alkaloids. Although compound **3**, homodeoxyharringtonine, has been shown to be present in cell cultures of the title plant by using GC−MS,[4] this is the first reported isolation from the natural source. We report herein the isolation and characterization of these alkaloids and their antileukemic activity against P-388 leukemia cells.

An alkaloidal extract was prepared in the usual way[5] from dried cut leaves and stems of the plant. It was chromatographed on ODS Si gel and reversed-phase preparative HPLC to yield **2**−**4** along with such known *Cephalotaxus* alkaloids as **1**.

Compound **1**, deoxyharringtonine, was identified by its spectroscopic data.[3] The assignments of its ^{1}H- and ^{13}C-NMR chemical shifts were made using 2D NMR techniques, and these assignments are listed in Tables 1 and 2 for comparison with those of the novel compounds.

Compound **2** was obtained as a pale yellowish oil. It gave positive I_2 and Dragendorff tests for alkaloids. HRFABMS of **2** gave a quasi molecular ion $[M + H]^+$ at *m*/*z* 502.2445, which is consistent with a molecular formula of $C_{27}H_{35}NO_8$. This formula sugggests that **2** lacks a methylene unit of **1**. The FABMS showed fragment ion peaks at *m*/*z* 298 (100) and 266 (35), which are characteristic for the cephalotaxine skeleton.[2] The IR spectrum showed the presence of hydroxyl groups (3530 cm^{-1}), an ester carbonyl group (1745 cm^{-1}), a carboxylic acid with intermolecular hydrogen bond (1652 cm^{-1}), and an aromatic ring (1490 cm^{-1}). In the ^{1}H-NMR spectrum ($CDCl_3$), four methyls (δ 0.82 d, 0.91 d, 3.57 s, and 3.67 s), seven sets of methylenes, a methine proton (δ 3.78 d), a vinyl proton (δ 5.04 s), a carbinyl proton (δ 5.97 d), methylene dioxide protons (δ 5.85 d and 5.88 d), and two aromatic protons (δ 6.53 s and 6.62 s) were observed. In the ^{13}C-NMR spectrum ($CDCl_3$), four methyls (δ 24.1 × 2, 51.5 and 57.1), eight methylenes, six methines, and seven quaternary and two carbonyl carbons (δ 170.5 and 174.4) were observed. These spectral data are very similar to those of **1** (Tables 1 and 2), except for the lack of a methylene unit in the diacid ester side chain. In the ^{1}H−^{1}H COSY spectrum, the partial structure −CH_2 (1″)−CH (2″)−Me_2 (3″ and 4″) was revealed. In the HMBC spectrum,[6] the H-3 proton was correlated with a carbonyl carbon C-1′, and



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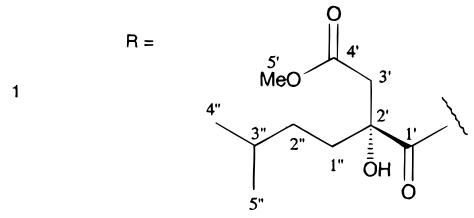

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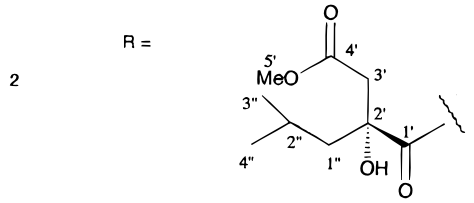

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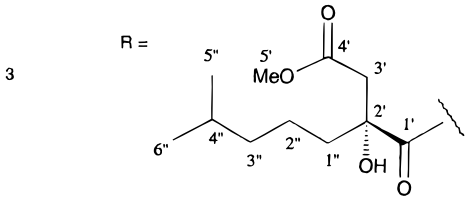

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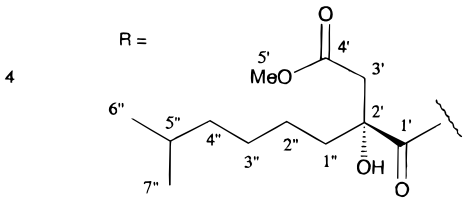

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Table 1. ^{1}H-NMR Spectral Data of **1**−**4**[a] (500 MHz, $CDCl_3$)

H	1	2	3	4
1	5.06 s	5.04 s	5.04 s	5.04 s
3	5.99 d (9.5)	5.97 d (9.8)	6.01 d (9.5)	5.99 d (9.5)
4	3.78 d (9.5)	3.78 d (9.8)	3.78 d (9.5)	3.78 d (9.5)
6α	2.05 m	2.03 dt (12.0, 10.0)	2.05 m	2.04 m
6*β*	1.95 m	1.90 ddd (12.0, 8.0, 4.0)	1.94 m	1.91 m
7α	1.79 m	1.76 m	1.77 m	1.76 m
7*β*	1.79 m	1.76 m	1.77 m	1.76 m
8α	3.15 m	3.10 m	3.10 m	3.10 m
8*β*	2.64 m	2.58 dd (9.0, 7.0)	2.59 m	2.60 m
10α	2.98 td (11.0, 7.0)	2.94 td (11.0, 7.0)	2.95 td (11.0, 7.0)	2.96 td (11.4, 7.0)
10*β*	2.64 m	2.60 dd (11.0, 8.0)	2.60 m	2.60 m
11α	2.40 dd (14.0, 6.8)	2.38 dd (14.0, 7.0)	2.38 dd (14.0, 7.0)	2.39 dd (13.9, 7.0)
11*β*	3.15 m	3.14 m	3.12 m	3.11 m
14	6.55 s	6.53 s	6.54 s	6.54 s
17	6.63 s	6.62 s	6.63 s	6.62 s
18a	5.86 d (1.5)	5.85 d (1.6)	5.85 d (1.4)	5.86 d (1.2)
18b	5.88 d (1.5)	5.88 d (1.6)	5.87 d (1.4)	5.87 d (1.2)
19	3.69 s	3.67 s	3.67 s	3.67 s
3′a	1.90 d (16.5)	1.83 d (16.3)	1.90 d (16.5)	1.93 d (16.5)
3′b	2.28 d (16.5)	2.24 d (16.3)	2.26 d (16.5)	2.27 d (16.5)
5′	3.57 s	3.57 s	3.57 s	3.57 s
1″a	1.41 m	1.34 dd (14.0, 6.0)	1.12 m	1.10−1.40 m
1″b	1.41 m	1.36 dd (14.0, 6.0)	1.40 m	1.10−1.40 m
2″a	0.97 m	1.65 m	1.38 m	1.10−1.40 m
2″b	1.28 m		1.38 m	1.10−1.40 m
3″a	1.41 m	0.82 d (6.7)	1.20 m	1.10−1.40 m
3″b			1.20 m	1.10−1.40 m
4″a	0.83 d (7.5)	0.91 d (6.7)	1.49 m	1.10−1.40 m
4″b				1.10−1.40 m
5″	0.84 d (7.5)		0.84 d (6.4)	1.50 m
6″			0.85 d (6.4)	0.85 d (6.9)
7″				0.87 d (6.9)

[a] Assignments from C/H correlation experiments. *J* values are given in Hz in parentheses.

Table 2. ^{13}C NMR Spectral Data of **1**−**4**[a] (125 MHz, $CDCl_3$)

C	1	2	3	4
1	99.9	100.0	100.1	100.0
2	157.9	157.8	157.8	157.8
3	74.5	74.8	74.5	74.6
4	55.8	55.9	55.8	55.8
5	70.7	70.6	70.7	70.6
6	43.3	43.4	43.4	43.3
7	20.2	20.3	20.3	20.3
8	53.9	54.0	53.9	53.9
10	48.5	48.7	48.5	48.6
11	31.2	31.3	31.2	31.3
12	133.1	133.4	133.4	133.3
13	128.3	128.4	128.3	128.4
14	112.6	112.7	112.6	112.7
15	146.6	146.7	146.7	146.7
16	145.8	145.8	145.8	145.8
17	109.6	109.7	109.6	109.7
18	100.8	100.8	100.8	100.0
19	57.1	57.1	57.3	57.2
1′	174.0	174.4	174.0	174.1
2′	74.7	75.2	74.7	74.7
3′	42.7	43.4	42.6	42.6
4′	170.4	170.5	170.5	170.5
5′	51.4	51.5	51.5	51.5
1″	36.7	46.7	39.0	38.8
2″	31.5	23.9	20.5	22.9
3″	27.9	24.1	38.9	38.8
4″	22.2	24.1	27.7	27.3
5″	22.6		22.4	27.8
6″			22.6	22.6
7″				22.7

[a] Assignments from C/H correlation experiments.

also the H-3′ methylene protons showed correlations with C-1′, C-2′, C-4′, and C-1″. This spectral evidence suggested that nordeoxyharringtonine (**2**) has an isobutyl branch at the C-2′ position in the side chain instead of an isopentyl branch found in **1**. The ^{1}H-NMR spectrum, NOESY correlations, and the CD spectrum of **2** were very similar to those of **1**. The vicinal coupling constant (J = 9.8 Hz) between H-3 and H-4 and the NOESY correlations of H-3 with H-4 showed that they were in the *cis* configuration. The NOESY correlations of H-4 with H-6α and H-14; H-1 with H6*β*, H-7*β*, H-8*β*, and H-10*β*; and H-11α with H-17 showed the ring conformation as the same as that of **1**. The CD spectrum of **2** was similar to that of **1**.[7] Furthermore, the molybdate complex of the diacid moiety derived from the acid hydrolysis of **2** showed a negative Cotton effect at 270 nm ([*θ*] −6800) indicating that the absolute configuration at C-2′ in the side chain moiety is *R*.[8−10] Thus, the absolute configuration of **2** is 3*S*, 4*S*, 5*R*, and 2′*R*.

Compounds **3** and **4** were obtained as pale yellowish oils. HRFABMS suggested a molecular formula of $C_{29}H_{39}NO_8$ for **3** and $C_{30}H_{41}NO_8$ for **4**. These formulae indicate, respectively, an excess of one and two methylene units over those found in **1**. In the ^{1}H- and ^{13}C-NMR ($CDCl_3$) spectra, the chemical shifts of **3** and **4** were very similar to those of **1** except for the side chain. The ^{1}H-NMR spectrum of **3** showed three sets of methylenes between *δ* 1.12 and *δ* 1.40 ppm, which integrated for six protons (H-1″a to H-3″b). The ^{1}H NMR of **4** showed a methylene envelope between *δ* 1.10 and *δ* 1.40, which integrated for eight protons (H-1″a to H-4″b). The ^{13}C-NMR spectrum showed 11 carbons for **3** and 12 carbons for **4** in the side chain. These carbons include two carbonyls, one carbomethoxyl group, two terminal methyl groups, one methine, and one carbinyl group. The ^{1}H−^{1}H COSY spectrum showed the partial structure $-CH_2$ (1″)$-CH_2$ (2″)$-CH_2$ (3″)$-$CH (4″)$-Me_2$ (5″ and 6″) for **3**, and $-CH_2$ (4″)$-$CH (5″)$-Me_2$ (6″ and 7″) for **4**. The HMBC spectra also indicated the correlations H-3 to C-1′; H-3′a to C-1′, C-2′, C-4′, and C-1″; H-3′b to

C-2′ and C-4′; H-5′ to C-4′ in **3**, and H-3 to C-1′ ; H-3′a to C-1′, C-2′, C-4′, and C-1″ ; H-3′b to C-2′ and C-4′ ; H6″ and H7″ to C5″ and C4″ for **4**. The above spectral evidence suggested that **3** has an isohexyl branch and **4** has an isoheptyl branch at the C-2′ position on the side chain moiety of **1**. The resemblance of the ^{1}H-NMR spectra, the NOESY correlations, and the CD spectra of **3** and **4** to those of **1**, and the negative Cotton effect at 270 nm (**3**: $[\theta]$ −6900, **4**: $[\theta]$ −1000) of the molybdate complex of the diacid moiety revealed the absolute configurations both **3** and **4** as 3*S*, 4*S*, 5*R*, and 2′*R*.

Because the acyl moiety of **1** is known to come from L-leucine by the same mechanism as that involved in the homologation of L-valine to L-leucine in microorganisms,[11,12] further homologation through this biosynthetic scheme would produce the acyl moiety of **3** and **4**, while the acyl moiety of **2** may come similarly from L-valine or directly from L-leucine.

The IC_{50} values of compounds **2**, **3**, and **4** against P-388 leukemia cells were 0.027, 0.056, and 0.024 μg/mL, respectively; these compounds are thus less cytotoxic than **1** (IC_{50} 0.0075 μg/mL).

Experimental Section

General Experimental Procedures. ^{1}H- and ^{13}C-NMR spectra were obtained in $CDCl_3$ with TMS as internal standard. NOESY experiments were performed with a mixing time of 0.40 s. FABMS was obtained in the positive ion mode. HPLC was performed with a CAPCELL PAK C_{18} UG 120A column (20 mm i.d. × 250 mm, Shiseido) packed with 5 μm ODS. TLC was conducted on precoated Kieselgel 60 F_{254} (Merck), and detection was achieved by UV at 254 nm, exposure to I_2 vapor, or spraying with Dragendorff's reagent, or combinations of these methods. NMR coupling constants (*J*) are given in Hz.

Plant Material. The leaves and stems of *C. harringtonia* var. *drupacea* were collected in Yamanashi Prefecture, Japan, in October 1994, and were identified by Dr. Susumu Isoda, Showa University. Voucher specimens are on deposit in the herbarium of the Tokyo Metropolitan Research Laboratory of Public Health, Tokyo, Japan.

Extraction and Isolation. Dried, cut leaves and stems (10 kg) of *C. harringtonia* var. *drupacea* were extracted with MeOH (3 × 50 L) at 70 °C for 5 days to give the extract (1.1 kg). Of the MeOH extract 800 g were suspended in 3% tartaric acid (16 L) and extracted with EtOAc (3 × 8 L), respectively. The aqueous phase was then basified with saturated aqueous Na_2CO_3 solution and extracted with $CHCl_3$ (3 × 8 L) . The $CHCl_3$-soluble phase was concentrated to give crude extract (16 g). The extract was suspended in H_2O (100 mL) and subjected to ODS column chromatography (1.6 kg). The column was conditioned with MeOH, H_2O, then aqueous 0.03 M $(NH_4)_2CO_3$ prior to use. Elution with aqueous 0.03 M $(NH_4)_2$ CO_3−MeOH mixtures of increasing MeOH concentration (0 to 100%) gave 13 fractions that were monitored by TLC and HPLC. Fractions obtained with 60% MeOH were combined and reprocessed on an ODS column or reversed-phase HPLC by aqueous 0.03 M $(NH_4)_2CO_3$−MeCN and/or aqueous 0.1 M NH_4OAc−MeCN solvent system to give **1** (185 mg), **2** (2 mg), **3** (44 mg), and **4** (4 mg).

Preparation of the Molybdate Complex of the Acid Moiety of 1−4. Of **1**−**4** 1 mg each was hydrolyzed with 1 mL of 3 M HCl (reflux, 4 days). After cooling, the reaction mixture was made basic with 3 M aqueous NH_3 and extracted with $CHCl_3$. Excess aqueous NH_3 was neutralized and evaporated under diminished pressure. The crude acids so obtained were used directly in the preparation of CD solutions. These solutions were 3.0 mM with respect to hydroxy acid and 2.7 mM with respect to sodium molybdate. HCl and NaOH solutions were added until pH 2.9−3.1 was reached. Measurements of the CD spectra were carried out in a 1-mm cell 5 days after the solutions had been prepared.

Nordeoxyharringtonine (2): a pale yellowish oil; $[\alpha]_D$ −90° (MeOH; *c* 0.07); FABMS *m*/*z* (rel int) 502 $[M + H]^+$ (50) , 298 $[M + H - C_9H_{16}O_5]^+$ (100) and 266 (35); HRFABMS *m*/*z* $[M + H]^+$ 502.2445, calcd for $C_{27}H_{36}NO_8$, 502.2441; IR ν_{max} (KBr) 3530 br, 2954, 2929, 2875, 2794, 1745, 1652, 1490 cm^{-1}; UV λ_{max} (MeOH) 291 (log ϵ 3.36) nm; CD (MeOH) $[\theta]_{291}$ −4500, $[\theta]_{261}$ 0, $[\theta]_{250}$ +2000, $[\theta]_{224}$ +14 300; ^{1}H NMR, Table 1; ^{13}C NMR, Table 2.

Homodeoxyharringtonine (3): a pale yellowish oil; $[\alpha]_D$ −122° (MeOH; *c* 1.00); FABMS *m*/*z* (rel int) 530 $[M + H]^+$ (100), 298 $[M + H - C_{11}H_{20}O_5]^+$ (100), 282 (20) and 266 (35); HRFABMS *m*/*z* $[M + H]^+$ 530.2758, calcd for $C_{29}H_{40}NO_8$, 530.2754; IR ν_{max} (KBr) 3529 br, 2953, 2926, 2854, 2797, 1750, 1653, 1505, 1488 cm^{-1}; UV λ_{max} (MeOH) 291 (log ϵ 3.55) nm CD (MeOH) $[\theta]_{291}$ −2100, $[\theta]_{262}$ 0, $[\theta]_{250}$ +1400, $[\theta]_{224}$ +7400; ^{1}H NMR, Table 1; ^{13}C NMR, Table 2.

Bishomodeoxyharringtonine (4): a pale yellowish oil; $[\alpha]_D$ −74° ($CHCl_3$; *c* 0.27); FABMS *m*/*z* (rel int) 544 $[M + H]^+$ (70), 298 $[M + H - C_{12}H_{22}O_5]^+$ (100) and 266 (35); HRFABMS *m*/*z* $[M + H]^+$ 544.2915, calcd for $C_{30}H_{42}NO_8$, 544.2910; IR ν_{max} (KBr) 3535 br, 2950, 2929, 2877, 2796, 1745, 1652, 1486 cm^{-1}; UV λ_{max} ($CHCl_3$) 293 (log ϵ 3.50) nm; CD (MeOH): $[\theta]_{291}$ −700, $[\theta]_{261}$ 0, $[\theta]_{250}$ +100, $[\theta]_{224}$ +6000; ^{1}H NMR, Table 1; ^{13}C NMR, Table 2.

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