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ESTER-TYPE CEPHALOTAXUS ALKALOIDS FROM *CEPHALOTAXUS HARRINGTONIA* VAR. *DRUPACEA*

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Key Word Index—*Cephalotaxus harringtonia* var. *drupacea*; Cephalotaxaceae; alkaloids; neoharringtonine; homoneoharringtonine; 3′*S*-hydroxyneoharringtonine; antileukaemic activities.

Abstract—Three alkaloids, neoharringtonine, homoneoharringtonine and 3′*S*-hydroxyneoharringtonine, were isolated from the leaves and stems of *Cephalotaxus harringtonia* var. *drupacea*. Their structures were established by spectroscopic methods, including two-dimensional NMR and CD spectra, and their antileukaemic activity was evaluated using P-388 leukaemia cells. Copyright © 1997 Elsevier Science Ltd

INTRODUCTION

The ester-type *Cephalotaxus* alkaloids, such as harringtonine (**1**), homoharringtonine (**2**), isoharringtonine (**3**) and deoxyharringtonine (**4**), have been isolated from *Cephalotaxus* species and their unique structure and potent antileukaemic activities have drawn the attention of many chemists [1]. Our recent search for antileukaemic substances in *C. harringtonia* var. *drupacea* led to the isolation of three minor ester-type *Cephalotaxus* alkaloids, neoharringtonine (**5**), homoneoharringtonine (**6**) and 3′*S*-hydroxyneoharringtonine (**7**). We report their structures and antileukaemic activities against P-388 leukaemia cells.

RESULTS AND DISCUSSION

The methanol extract from dried cut leaves and stems was partitioned between ethyl acetate and 3% tartaric acid. The acidic aqueous phase was then made basic, to pH 8.5, by the addition of saturated aqueous sodium carbonate solution and extracted with chloroform. The crude chloroform extract was chromatographed on ODS silica gel and reverse-phase preparative HPLC, which yielded compounds **5**–**7**, along with known *Cephalotaxus* alkaloids, e.g. compounds **1**–**4**.

Compound **5** had the molecular formula $C_{30}H_{33}NO_8$, established by high-resolution FAB-mass spectrometry, indicating 15 degrees of unsaturation. Spectral data revealed the presence of a hydroxyl group (3520 cm^{-1}), two methoxyl groups (δ_H 3.55, δ_C 51.6 and δ_H 3.71, δ_C 57.2), an aromatic ring with two *para*-coupling protons (δ_H 6.52, δ_C 112.9 and δ_H 6.64, δ_C 109.7) and a methylenedioxy group (δ_H 5.80, 5.86; δ_C 100.8), which are characteristic of the ring system of *Cephalotaxus* alkaloids. In addition, two AB type methylenes (δ_H 1.90, 2.23; δ_C 41.7 and δ_H 2.70, 2.71; δ_C 44.3), two ester carbonyl (δ_C 170.4 and 173.6) and five aromatic methine signals (δ_H 7.14 × 2, 7.21 × 2, 7.23; δ_C 130.6 × 2, 127.9 × 2, 126.9) indicated that compound **5** had an ester side-chain with a terminal phenyl group. Taking its cyclic system and an ester side-chain moiety into consideration, HMBC [2] correlations between the proton signals of the above structural fragments and nine quaternary carbon signals, including sp^3 carbons at δ_C 70.5, 74.9, 128.3, 133.4, 145.8, 146.7, 157.6, 170.4 and 173.6 could assemble the planar structure. The NOESY correlations of H-1 with H-6β, H-7β, H-8β and H-10β; H4 with H-3, H-6α and H-14; H-8α with H-10α suggested the ring formation of compound **5** as shown in Fig. 1. Although this compound had been reported as neoharringtonine from *C. hainanensis* [3], this is the first report of its occurrence in *C. harringtonia*.

Compound **6** was obtained as a pale yellowish oil. The high-resolution FAB-mass spectrum suggested a molecular formula of $C_{31}H_{55}NO_8$, which indicated the presence of an additional methylene unit compared with compound **5**. In the ^1H and ^{13}C NMR ($CDCl_3$) spectra, the chemical shifts of compound **6** were very similar to those of compound **5**, except around the side-chain moiety. In the ^1H NMR, one of the AB-

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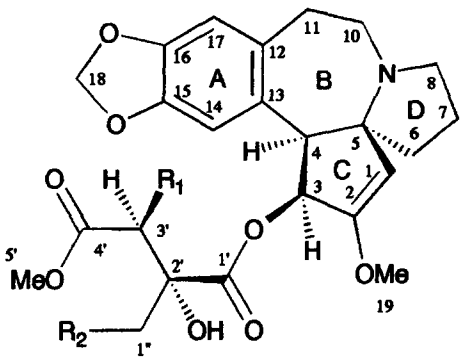


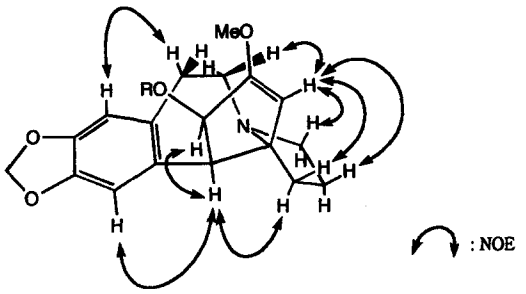
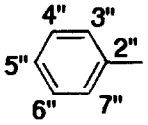
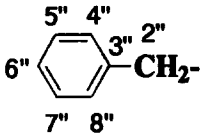
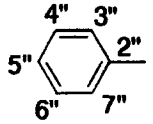


Fig. 1. Selected NOESY correlations commonly observed for the ring moiety of compounds **5**–**7**.

	R_1	R_2
1	H	$HO(Me)_2C\text{-}CH_2\text{-}$
2	H	$HO(Me)_2C\text{-}CH_2\text{-}CH_2\text{-}$
3	OH	$(Me)_2CH\text{-}CH_2\text{-}$
4	H	$(Me)_2CH\text{-}CH_2\text{-}$
5	H	
6	H	
7	OH	

type methylene signals (δ 2.70 and 2.71) of compound **5** had disappeared and two sets of methylenes between δ 1.75 and 2.62 (H-1″ab and H-2″ab) were observed. The ^{13}C NMR showed 13 carbons on the side-chain moiety, which were two carbonyl, one methoxyl, one phenyl, three methylene and one carbinyl group. The ^1H–^1H COSY spectrum of compound **5** showed the partial structure CH_2(1″)-CH_2(2″)- and CH(4″)-CH(5″)-CH(6″)-CH(7″)-CH(8″)-. The HMBC spectrum indicated that the correlations from 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′ and from H-2″ to C-3″. The above spectral evidence suggested that compound **6** has a phenethyl branch at the C-2′ position on the side-chain moiety. Although this compound had been semi-synthesized from cephalotaxine and γ-phenyl-α-butanoic acid [4], this is the first isolation from a natural source. The isolation of alkaloids **5** and **6** is also informative regarding their biosynthesis. Since the acyl moiety of **4** has been known to be biosynthesized from L-leucine by a similar mechanism to that involved in the homologation of L-valine to L-leucine in microorganisms, or directly from homoleucine [5, 6], the acyl moiety of compound **5** could be biosynthesized from L-phenylalanine. Further homologation through this biosynthetic scheme would produce the acyl moiety of **6**.

Compound **7** was obtained as oil. The high-resolution high resolution FAB-mass spectrum suggested a molecular formula of $C_{30}H_{33}NO_9$. In the ^1H and ^{13}C NMR spectra, the chemical shifts of compound **7** were in good agreement with those of compound **5**, except around the C-3′ position. Although the H-3′ protons of compound **5** resonated at δ 1.90 and δ 2.23 as an AB-type doublet (J = 16.5 Hz), the proton assignable to this position was observed at δ 3.34 as a broad doublet in compound **7**. In addition, the C-3′ resonance was observed at lower field ($\Delta\delta$ +32.4) than compound **5**, which suggested the presence of a hydroxyl group at the C-3′ position. Thus, compound **7** was revealed as the 3′-hydroxyl derivative of compound **5**. A vicinal coupling (J = 9.8 Hz) between H-3 and H-4, the NOESY correlations of H-1 with H-6β, H-7β, H-8β and H-10β; H-4 with H-3, H-6α and H-14; H-11α with H-17, suggested that compound **7** also had the same ring configuration as compound **5** (Fig. 1).

The absolute configurations of compounds **5**–**7** were established by CD spectra. Methanol solutions of compounds **5**–**7** all showed negative Cotton effects (**5** $[\theta]_{290}-1500$; **6** $[\theta]_{290}-3100$; and **7** $[\theta]_{290}-2600$. They were similar to those of alkaloids **2**–**4** (**2** $[\theta]_{29}-3600$; **3** $[\theta]_{290}-2800$; and **4** $[\theta]_{290}-2600$), whose absolute configurations are known. This indicated that the chirality between the two chromophores (the aromatic A ring and the double bond on the C ring) of compounds **5**–**7** were the same as those of compounds **2**–**4** (3*S*, 4*S*, 5R). Voelter *et al.* reported that CD spectra of α-hydroxyacids give valuable information for the determination of their absolute configurations [7]. Brandänge *et al.* applied this method for some C-2′ alkyl diacids and found that the negative Cotton effect at 270 nm of the molybdate complex were the characteristic sign of the 2′R-configuration, and they determined the absolute configuration of the side-chain moiety of compound **4** [8, 9]. The CD spectra for the

Table 1. ^{1}H NMR spectral data of compounds **5**–**7*** (500 MHz, $CCDl_3$).

H	5	6	7
1	5.07*s*	5.09*s*	5.11*s*
3	5.86*d* (9.8)	6.07*d* (9.8)	5.93*d* (9.8)
4	3.77*d* (9.8)	3.81*d* (9.8)	3.77*d* (9.8)
6α	2.01*td* (12.0, 9.5)	2.05 *td* (12.0, 9.7)	2.03 *td* (12.5, 9.8)
6β	1.89*ddd* (12.0, 8.0, 4.0)	1.93 *ddd* (12.4, 8.3, 4.4)	1.90*ddd* (12.0, 8 .0, 4.2)
7α	1.74*m*	1.75*m*	1.77*m*
7β	1.74*m*	1.75*m*	1.77*m*
8α	3.09*td* (12.0, 7.0)	3.12*td* (8.5, 5.0)	3.12*m*
8β	2.59*m*	2.62*m*	2.61*m*
10α	2.93*td* (12.0, 7.0)	2.95*td* (12.0, 7.0)	2.97*td* (12.0, 7.0)
10β	2.59*m*	2.62*m*	2.61*m*
11α	2.40*dd* (14.0, 7.0)	2.37*m*	2.41*dd* (14.0, 7.0)
11β	3.17*ddd* (14.0, 12.0, 8.0)	3.13*m*	3.12*m*
14	6.52*s*	6.56*s*	6.51*s*
17	6.64*s*	6.59*s*	6.68*s*
18a	5.80*d* (1.5)	5.73*d* (1.5)	5.81*d* (1.5)
18b	5.86*d* (1.5)	5.84*d* (1.5)	5.88*d* (1.5)
19	3.71*s*	3.70*s*	3.74*s*
3′a	1.90*d* (16.5)	1.97*d* (16.5)	3.34*brd* (7.0)
3′b	2.23*d* (16.5)	2.30*d* (16.5)	
5′	3.55*s*	3.59*s*	3.64*s*
1″a	2.70*d* (13.5)	1.75*m*	2.84*d* (13.9)
1″b	2.71*d* (13.5)	1.75*m*	3.17*d* (13.9)
2″a	—	2.37*m*	—
2″b	—	2.62*m*	—
3″	7.14*m*	—	7.21*m*
4″	7.21*m*	7.11*br d* (8.0)	7.21*m*
5″	7.23*m*	7.27*br t* (8.0)	7.21*m*
6″	7.21*m*	7.18*br t* (7.5)	7.21*m*
7″	7.14*m*	7.27*br t* (8.0)	7.21m
8″	—	7.11*br d* (8.0)	—

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

molybdate complex of the diacid derived from the hydrolysis of compounds **5** and **6** both showed negative Cotton effects at $[\theta]_{268}-11\,200$ for **5** and $[\theta]_{268}-5000$ for **6**, which were in good agreement with those of compounds **2** ($[\theta]_{270}-3400$) and **4** ($[\theta]_{270}-12\,800$). This indicated that the absolute configurations at C-2′ of both compounds **5** and **6** are 2′R. Furthermore, Brandänge *et al.* also applied this method for compound **3** and suggested that the negative Cotton effect at 258 nm of the molybdate complex was the characteristic sign of the 2′*R*3′*S* configuration [10]. The CD spectra for the molybdate complex of the diacid moiety derived from the hydrolysis of compound **7** showed a negative Cotton effect at 256 nm ($[\theta]-1800$), which was similar to that of compound **3** ($[\theta]_{258}-1400$). Thus, the absolute configuration of the side-chain moiety of compound **7** was revealed as 2′*R*3′*S*. Therefore, the absolute configurations of compounds **5**–**7** were 3*S*, 4*S*, 5*R* and 2′*R* for **5**, 3*S*, 4*S*, 5*R* and 2′*R* for **6** and 3*S*, 4*S*, 5*R*, 2′*R* and 3′*S* for **7**.

Compounds **5**–**7** showed antileukaemic activities against P-388 leukaemia cells, and their IC_{50} values were 0.012, 0.28 and 0.19 μg ml^{-1}, respectively. Compound **5** was ten times more active than compounds **6** and **7**, indicating that the antileukaemic activity of this type of alkaloid is affected by variations in the composition of the side-chain moiety.

EXPERIMENTAL

General. ^{1}H and ^{13}C NMR: $CDCl_3$ with TMS as int. standard. NOESY were conducted with a mixing time of 0.40 sec. FAB-MS: (positive). HPLC was performed with a CAPCELL PAK C18 UG 120A column (20 mm i.d. × 250 mm, Shiseido) packed with 5 μm ODS. TLC was conducted on precoated Kieselgel 60 F_{254} and detection was achieved by UV light at 254 nm, exposure to I_2 vapour and/or spraying with Dragendorff's reagent.

Materials. Leaves and stems of *C. harringtonia* var. *drupacea* (Sieb. & Zucc.) Koidzumi were collected in Yamanashi Prefecture, Japan, in October 1994, and identified by Dr Susumu Isoda (Showa University). Voucher specimens are deposited in the Herbarium of the Tokyo Metropolitan Research Laboratory of Public Health, Tokyo, Japan.

Extraction and isolation. Dried cut leaves and stems (10 kg) were extracted with MeOH (3 × 50 l) at 70° for 5 days to give the extract (1.1 kg). The MeOH extract

Table 2. ^{13}C NMR spectral data of compounds **5–7*** (125 MHz, $CDCl_3$)

C	5	6	7
1	100.3	100.3	100.7
2	157.6	157.6	157.4
3	75.2	74.9	75.6
4	55.9	55.9	55.9
5	70.5	70.6	70.5
6	43.3	43.4	43.4
7	20.2	20.3	20.2
8	53.9	54.0	53.9
10	48.6	48.7	48.5
11	31.4	31.4	31.3
12	133.4	133.3	133.6
13	128.3	125.9	128.2
14	112.9	112.6	112.9
15	146.7	146.7	146.8
16	145.8	145.8	145.6
17	109.7	109.7	109.9
18	100.8	100.8	100.9
19	57.2	57.3	57.2
1′	173.6	173.8	172.4
2′	74.9	74.6	79.2
3′	41.7	42.7	74.1
4′	170.4	170.3	171.6
5′	51.6	51.6	52.5
1″	44.3	41.0	40.5
2″	134.8	29.3	134.8
3″	130.6	141.8	130.7
4″	127.9	128.4	127.9
5″	126.9	128.3	126.9
6″	127.9	125.9	127.9
7″	130.6	128.3	130.7
8″		128.4	

* Assignments from C/H correlation experiments.

(800 g) was suspended in 3% tartaric acid (16 l) and extracted with EtOAc (3 × 8 l). Then, the aq. phase was made alkaline with aq. Na_2CO_3 soln and extracted with $CHCl_3$ (3 × 8 l). The $CHCl_3$-sol. phase was concd to give a crude extract (16 g). This was suspended in H_2O (100 ml) and subjected to ODS CC (1.6 kg) conditioned with MeOH, H_2O, then 0.03 M aq. $(NH_4)_2CO_3$. Using 0.03 M aq. $(NH_4)_2CO_3$–MeOH mixts of increasing MeOH concn (0–100%), 13 frs were obtained which were monitored by TLC and HPLC. Frs were combined and processed on ODS column or reverse RP-HPLC using 0.03 M aq. $(NH_4)_2CO_3$–MeCN solvent systems, to give compounds **1** (108 mg), **2** (2410 mg), **3** (297 mg) and **4** (185 mg), **5** (22 mg), **6** (1 mg) and **7** (4 mg).

Preparation of molybdate complexes. **2–7** (1 mg) were hydrolysed with 1 ml of 3 M HCl (reflux, 4 days). After cooling, the mixt. was made basic with 3 M NH_4OH and the akaline phase washed with $CHCl_3$. Excess NH_4OH was neutralized and evapd under red. pres. The crude acids so obtained were used directly in the prepn of CD solns, which were 3 mM with respect to hydroxy acids and 2.7 mM with respect to Na molybdate. HCl and NaOH solns were added until pH 2.9–3.1 was reached. Measurements of CD were carried out in a 1 mm cell 5 days after the solns had been prepd.

Neoharringtonine (5). Oil. $[\alpha]_D$ −148° (MeOH; *c* 0.48). FABMS *m/z* (rel. int.): 536 $[M+H]^+$ (74), 298 (100), 284 (20). (HRFAB-MS, found: $[M+H]^+$, 536.2268. $C_{30}H_{34}NO_8$ requires $[M+H]^+$, 536.2284). IR ν_{max}^{KBr} cm^{-1}: 3520 *br*, 2954, 2920, 2880, 2796, 1746, 1653, 1503, 1488, 1456, 1439, 1365, 1343. UV λ_{max}^{MeOH} nm: 291 (log ε 3.61). ^1H NMR: Table 1. ^{13}C NMR: Table 2.

Homoneoharringtonine (6). Oil. $[\alpha]_D$ −114° (MeOH; *c* 0.07). FAB-MS *m/z* (rel. int): 550 $[M+H]^+$ (67), 316 (11), 298 (100), 284 (36). (HRFAB-MS, found: $[M+H]^+$, 550.2432. $C_{31}H_{36}NO_8$ requires $[M+H]^+$, 550.2440). IR ν_{max}^{KBr} cm^{-1}: 3500 *br*, 2953, 2924, 2856, 2792, 1745, 1652, 1503, 1487, 1455, 1439, 1365, 1343. UV λ_{max}^{MeOH} nm: 291 (log ε 3.39). ^1H NMR: Table 1. ^{13}C NMR: Table 2.

3′*S*-Hydroxyneoharringtonine (7). Oil. $[\alpha]_D$ −126° (MeOH; *c* 0.20). FAB-MS *m/z* (rel. int): 552 $[M+H]^+$ (20), 314 (11), 298 (100), 282 (20), 266 (25). (HRFAB-MS, found: $[M+H]^+$, 552.2227. $C_{30}H_{34}NO_9$ requires $[M+H]^+$, 552.2234). IR ν_{max}^{KBr} cm^{-1}: 3515 *br*, 2955, 2880, 2792, 1743, 1652, 1487, 1448, 1365, 1343. UV λ_{max}^{MeOH} nm: 291 (log ε 3.66). ^1H NMR: Table 1. ^{13}C NMR: Table 2.

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