New Alkaloids from *Cephalotaxus fortunei*

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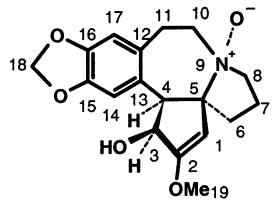
Four new *cephalotaxus* alkaloids, cephalotaxine α-*N*-oxide (**1**), cephalotaxine *β*-*N*-oxide (**2**), 11-*β*-hydroxycephalotaxine *β*-*N*-oxide (**3**), and isocephalotaxine (**4**), were isolated, together with several known alkaloids from an EtOAc extract of the fruits of *Cephalotaxus fortunei*. The structures were determined by spectral analysis including mass spectrometry and 2D NMR. Compounds **1**, **2**, **3**, and **4** displayed cytotoxicity against nasopharynx KB cells with IC_{50} values of 30, 14, 31, and 15 *μ*g/mL, respectively.

The genus *Cephalotaxus*, evergreen trees of southern Asia, contains cephalotaxine ester type alkaloids, harringtonine, and related cytotoxic substances. Clinical trials in the People's Republic of China and elsewhere have demonstrated that harringtonine, homoharringtonine, and homodeoxyharringtonine are effective in the treatment of acute leukemia. Thus, intensive investigations have been made to discover other antitumor cephalotaxus alkaloids.[1−7]

Investigating the seeds of *Cephalotaxus fortunei*, Hook, f. (Cephalotaxaceae), we isolated four new minor alkaloids: cephalotaxine α-*N*-oxide (**1**), cephalotaxine *β*-*N*-oxide (**2**), 11-*β*-hydroxcephalotaxine *β*-*N*-oxide (**3**), and isocephalotaxine (**4**). In this paper, we report the isolation, structure elucidation, and cytotoxicity of these alkaloids against KB cells.

The methanol extract of seeds of *C. fortunei* was partitioned between ethyl acetate and 5% hydrochloric acid. The aqueous phase was made basic by ammonium hydroxide and extracted with ethyl acetate. The crude alkaloid fraction, obtained from the organic layer, was subjected to repeated chromatography on silica gel and furnished new compounds **1**−**4** together with the known alkaloids cephalocyclidin A,[8] cephalotaxine,[9] wilsonine, drupacine, 11-hydroxycephalotaxine,[10] cephalotaxinone, and demethylcephalotaxinone and the esters harringtonine, deoxyharringtonine, and isoharringtonine.[1,2]

Products **1** and **2** were amorphous solids with optical activity respectively $[\alpha]^{21}_D$ −131° and −221°. The HR-FABMS of **1** and **2** showed the protonated molecular ion $[MH]^+$ at *m*/*z* 332.1481 and 332.1515, and the same molecular formula ($C_{18}H_{21}NO_5$) was deduced for both compounds. They both had one oxygen atom more than cephalotaxine and displayed similar ^1H and ^{13}C NMR spectra (Tables 1 and 2). The same cephalotaxine-like structure was evident from analysis of the 2D NMR spectra, both compounds showing typical signals of two *para* protons H-14 and H-17 of a phenyl ring bearing a methylenedioxy, a methoxy (C-19), and two methylenes (C10 and C-11) in an AABB system and a methine (H-4) adjacent to a hydroxymethine (H-3). The $^1H-^1H$ COSY spectrum also confirmed the presence of three vicinal methylene groups (C-6, C-7, and C-8), forming a pyrrolidine ring and characteristic of the cephalotaxine skeleton. The strongly downfield shifted signal of the quaternary sp^3 carbon (C-5) at *δ* 85.4 and 89.1 respectively for **1** and **2**, compared with that of cephalotaxine (*δ* 70.4), allowed us to assign the *N*-oxide structure to compounds **1** and **2**. In fact, C-8 and C-10 were also influenced by the *N*-oxide group. Hence **1** and **2** must be stereoisomers.



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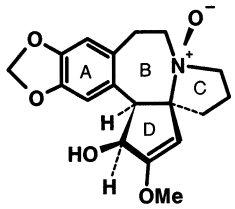

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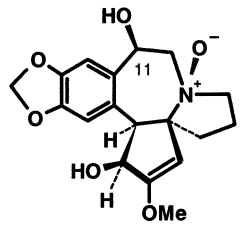

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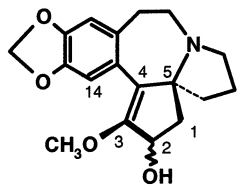

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The configurations of **1** and **2** were established by analysis of the NOESY correlations. Strong NOEs was observed between H-1 and H-10b for **1** and H-8a and H-11a for **2** (Figure 1). The structures obtained by molecular modeling with MM force field calculations[11] indicated that H-1 was very close to H-10b on the *β* side of cephalotaxine α-*N*-oxide **1** and H-8a was situated near by H-11a on the α side of *β*-*N*-oxide **2**. This is in agreement with a previous synthetic study of homoharringtonine *N*-oxides, indicating that the C-5 chemical shift was at higher field for the α-*N*-oxide (*δ* 85) than for the *β*-*N*-oxide (*δ* 89).[12] The location of the nitrogen lone electron pair in **1** and **2** induced deep changes in the B, C, and D ring conformations of cephalotaxine. This was evident in NMR spectra of compounds **1** and **2**, especially from differences of ^{13}C shifts and NOEs (Figure 1). The coupling constant between H-3 and H-4 also varied from 9.2 Hz in α-*N*-oxide **1** to 4.3 Hz in *β*-*N*-oxide **2**, due to a change of the dihedral angle H−C3−C4−H from 8° for **1** to 43° for **2**. Confirmation of structures was provided by synthesis of **1** and **2** from cephalotaxine by oxidation with hydrogen peroxide in methanol. The absolute configurations of **1** and **2** were thus determined as 3*S*, 4*S*, 5*R*, and *NR* for α-*N*-oxide **1** and *NS* for *β*-*N*-oxide **2**.

The ESI-TOFMS of compound **3** displayed the protonated molecular ion $[MH]^+$ at *m*/*z* 348.1440, indicating the

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Table 1. ^{1}H NMR Data (δ_H) of **1**−**4** in $CDCl_3$−10% CD_3OD (400 MHz)[a]

position	1	2	3	4
1	4.65 s	4.70 s	4.79 s	2.84 m
				1.81 m
2				5.12 m
3	4.62 s (9.2)	4.00 d (4.3)	4.21 d (6.9)	
4	3.64 d (9.2)	3.32 d (4.3)	3.51 d (6.9)	
6a	2.64 m	2.11 m	1.86 m	1.87 m
6b	1.83 m	2.11 m	2.51 m	1.79 m
7a	1.78 m	1.91 m	1.55 m	1.98 m
7b	2.02 m	2.14 m	2.08 m	2.01 m
8a	3.33 m	3.28 m	3.08 m	2.89 m
8b	3.44 m	3.58 m	3.21 m	3.62 m
10a	3.50 m	3.54 m	3.93 dd (14.4, 8.4)	3.46 m
10b	3.45 m	3.29 m	3.68 dd (14.4, 7.5)	2.86 m
11a	2.31 m	2.38 m	4.73 dd (8.4, 7.5)	2.81 m
11b	3.52 m	4.03 m		3.08 m
14	6.49 s	6.48 s	6.49 s	6.78 s
17	6.52 s	6.48 s	6.76 s	6.51 s
18	5.74 d (1.4)	5.78 d (1.4)	5.84 d (1.3)	5.87 s
	5.76 d (1.4)	5.82 d (1.4)	5.86 d (1.3)	5.87 s
19	3.63 s	3.66 s	3.68 s	3.74 s

[a] Coupling constants (Hz) in parentheses.

Table 2. ^{13}C NMR Data (δ_C) of **1**−**4** in $CDCl_3$−10% CD_3OD (75 MHz)

position	1	2	3	4
1	97.1	97.8	98.2	44.6
2	164.9	169.1	166.0	69.0
3	71.7	77.3	74.0	160.2
4	53.8	57.7	57.9	111.1
5	85.4	89.1	92.0	80.1
6	39.1	29.4	34.8	34.5
7	17.9	16.9	19.5	20.6
8	70.2	68.8	64.3	51.3
10	62.1	62.8	62.8	49.5
11	28.5	29.3	69.4	30.3
12	131.1	133.5	132.9	129.9
13	128.8	126.5	125.0	124.5
14	111.3	112.5	111.7	111.3
15	145.5	146.1	147.9	146.1
16	145.5	146.6	147.9	147.4
17	109.4	109.8	111.4	108.7
18	100.6	100.9	101.4	101.1
19	57.4	56.7	57.1	57.1

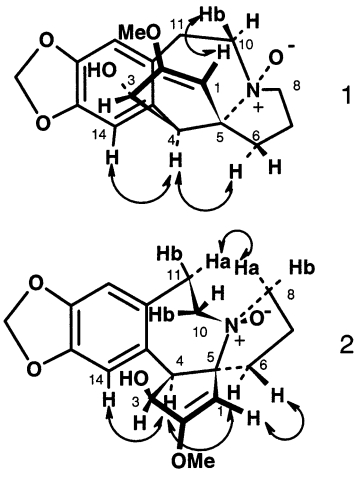

Figure 1. Selected NOE interactions for compounds **1** and **2**.

molecular formula $C_{18}H_{21}NO_6$. The ^{1}H, ^{13}C, and 2D NMR spectra (Tables 1 and 2) showed typical cephalotaxine skeleton signals: two *para* protons H-14 (δ 6.49) and H-17 (δ 6.76) of a phenyl ring bearing a methylenedioxy, a methoxy (C-19), three methylenes at positions 6, 7, and 8 of the pyrrolidine ring, a methine (H-4, δ 3.51) vicinal to hydroxymethine (H-3, δ 4.21), and a sp^2 methine (CH-1, δ_H 4.79, δ_C 98.2) composing a cyclopentene ring. The downfield shifted sp^3 quaternary carbon (C-5) at δ 92.0 suggested an *N*-oxide group. A hydroxymethine proton (δ_H 4.73) was observed as part of an ABX spin system with two methylene protons at C-10 (δ 3.68 and 3.93). Hence, compound **3** was 11-hydroxycephalotaxine *N*-oxide. In the NOESY spectrum, H-8a was correlated with H-11 and H-1 with H-6b. These interactions indicated that the β-*N*-oxide configuration of **3** was similar to compound **2**, locating H-11 on the α side and the hydroxy group on the β side. The structure of **3** was thus established as 11-β-hydroxycephlotaxine β-*N*-oxide.

Isocephalotaxine **4** was isolated as an optically active amorphous solid, $[\alpha]^{21}_D$ −47°. The ESI-TOFMS of **4** showed the protonated molecular ion $[MH]^+$ at *m*/*z* 316.1544, suggesting formula $C_{18}H_{21}NO_4$, in agreement with ^{13}C NMR data. The ^{1}H, ^{13}C, and 2D NMR spectra (Tables 1 and 2) were similar to those of cephalotaxine, showing a tetrahydrobenzazepine ring system bearing a methylenedioxy group, and condensed to a pyrrolidine and a cyclopentene ring. The main differences between the two compounds concerned the substitution pattern of the cyclopentene ring with the methoxyl and hydroxy groups, as well as the location of the carbon−carbon double bond.

In the ^{1}H−^{1}H COSY spectrum, the hydroxymethine proton at δ 5.12 (C-2) was correlated with the nonequivalent methylene protons (C-1) at δ 2.84 and 1.81, forming an ABX system. The HMBC spectrum showed long-range correlations of the quaternary sp^2 carbon at δ 111.1 with the two protons at C-1, with the proton at C-2, and also with the aromatic proton (C-14) at δ 6.78 and was thus assigned to C-4. The sp^2 carbon at δ 160.2 (C-3) was correlated to both the hydroxymethine H-2 and the methylene 2H-1, situating C-3. The quaternary sp^3 carbon at δ 80.1, linked to the nitrogen atom, gave correlations with protons H-2, H-8, H-10 (δ 3.46), and H-6 (δ 1.87) and was assigned to C-5, forming the cyclopentene and pyrrolidine rings. Compound **4** was thus an isomer of cephalotaxine. The methoxyvinyl and the hydroxymethine groups are reversed in alkaloid **4**, placing the carbon−carbon double bond at the 3−4 position instead of the 2−3 position in cephalotaxine and the hydroxymethine at the 2 position. The stereochemistry of the hydroxy methine at C-2 remains unknown, due to the absence of significant interaction in the NOESY spectrum. However, the results led to the structure of isocephalotaxine for this natural product **4**. This alkaloid was previously synthesized from isocephalotaxinone.[13]

The new alkaloids displayed weak cytotoxicity against human nasopharynx carcinoma KB cells with IC_{50} values of 30 (**1**), 14 (**2**), 31 (**3**), and 15 (**4**) μg/mL.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a Perkin-Elmer 341 instrument. Mass spectra were obtained either on ZAB2-SEQ (VG analytical) for the FABMS or on API Q-STAR (Applied Biosystem) for the ESI-TOFMS. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AC 300 spectrometer (^{1}H: 300.13; ^{13}C: 75.43 MHz), respectively, or on an Avance 400 Bruker (^{1}H: 400.13; ^{13}C: 100.61 MHz) with internal reference (^{1}H: CHD_2OD at δ 3.31 or $CHCl_3$ at δ 7.26; ^{13}C: CD_3OD at δ 49.0 and $CDCl_3$ at δ 77.0).

Plant Material. The fruits were collected in September 1998 from a *C. fortunei* tree grown in the Arboretum at Chèvreloup (Muséum National d'Histoire Naturelle). A voucher specimen was deposited at the Herbarium of the MNHN, Paris.

Extraction and Isolation. The drupes (fruits) of *C. fortunei* (5 kg) were ground, extracted with MeOH (20 L) four

times, and concentrated to dryness under reduced pressure. The oily residue was partioned between aqueous HCl (5%) and EtOAc. The aqueous layers were basified with aqueous ammonia and then extracted with EtOAc. The organic layer was dried over Na_2SO_4 and concentrated to dryness to yield 19 g of crude alkaloids.

The extract was subjected to chromatography on a silica gel column eluting with CH_2Cl_2−MeOH mixtures, with increasing MeOH concentration (5 to 100%). The polar fractions combined were rechromatographed on silica gel to give **1** (50 mg), **2** (132 mg), **3** (18 mg), **4** (5 mg), cephalocyclidin A (40 mg), cephalotaxine (5.2 g), wilsonine (1.2 g), drupacine (4.9 g), 11-hydroxycephalotaxine (6 mg), cephalotaxinone (100 mg), and demethylcephalotaxinone (10 mg) and esters harringtonine (98 mg), deoxyharringtonine (30 mg), and isoharringtonine (9 mg).

Cephalotaxine α-*N*-oxide (1): amorphous solid; $[\alpha]^{21}_D$ −131° (*c* 0.5, $CHCl_3$); ^{1}H and ^{13}C NMR, Tables 1 and 2; CIMS (NH_3) *m*/*z* 332 $[MH]^+$ (100), 314 (25), 217 (6), 141 (11); FABMS *m*/*z* 332 $[MH]^+$ (9), 316 (100), 314 $[MH - H_2O]^+$ (99), 298 $[MH - 2H_2O]^+$ (51); HRFABMS *m*/*z* 332.1481 (calcd for $C_{18}H_{22}NO_5$, 332.1498).

Cephalotaxine *β*-*N*-oxide (2): amorphous solid; $[\alpha]^{21}_D$ −221° (*c* 0.5, $CHCl_3$); ^{1}H and ^{13}C NMR, Tables 1 and 2; CIMS (NH_3) *m*/*z* 332 $[MH]^+$ (100), 316 (40), 314 (25), 217 (6), 171 (3), 141 (14); FABMS *m*/*z* 332 $[MH]^+$ (14), 316 (100), 314 $[MH - H_2O]^+$ (65), 298 $[MH - 2H_2O]^+$ (66); HRFABMS *m*/*z* 332.1515 (calcd for $C_{18}H_{22}NO_5$, 332.1498).

11-*β*-Hydroxycephalotaxine *β*-*N*-oxide (3): amorphous solid; $[\alpha]^{21}_D$ −94° (*c* 0.5, $CHCl_3$); ^{1}H and ^{13}C NMR, Tables 1 and 2; CIMS (NH_3) *m*/*z* 348 $[MH]^+$ (100), 332 (27), 330 (22), 314 (5), 180 (10), 163 (7); ESI-TOFMS *m*/*z* 348.1440 (calcd for $C_{18}H_{22}NO_6$, 348.1445).

Isocephalotaxine (4): amorphous solid; $[\alpha]^{21}_D$ −47° (*c* 0.5, $CHCl_3$); ^{1}H and ^{13}C NMR, Tables 1 and 2; CIMS (NH_3) *m*/*z* 316 $[MH]^+$ (88), 298 $[MH - H_2O]^+$ (100), 180 (26), 163 (29), 141 (14); ESI-TOFMS *m*/*z* 316.1544 (calcd for $C_{18}H_{22}NO_4$, 316.1549).

Synthesis of Cephalotaxines α-*N*-oxide (1) and *β*-*N*-oxide (2). A mixture of cephalotaxine (100 mg) in MeOH (5 mL) and 30% aqueous H_2O_2 (1 mL) was stirred at room temperature for 24 h. The solvent was evaporated, and the residue was submitted to silica gel column chromatography (CH_2Cl_2−MeOH, 85:15) to provide α-*N*-oxide **1** (43 mg) and *β*-*N*-oxide **2** (38 mg). The natural and synthetic compounds were identical by ^{1}H and ^{13}C NMR, MS, and $[\alpha]_D$.

Cytotoxic Activity. The cytotoxicity assays were carried out in 96-well microtiter plates, in triplicate, against human nasopharynx carcinoma KB cells (10^4 cells/mL). Cell growth was estimated by colorimetric measurement of stained living cells by neutral red. Optical density was determined at 540 nm on a Titertek Multiskan photometer after 72 h incubation.

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