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SEÑORES
DIVISIÓN DE NUEVAS CREACIONES
SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
E. S. D.

COD:08

RE.: SOLICITUD DE PATENTE EN COLOMBIA

EXPEDIENTE Nº	**00-00788**
TITULO:	AISLAMIENTO Y PURIFICACIÓN DE PACLITAXEL Y OTROS TAXANOS MEDIANTE CROMATOGRAFÍA DE BAJA PRESIÓN DE PREPARACIÓN INDUSTRIAL EN UNA COLUMNA DE RESINA POLIMÉRICA.
SOLICITANTE:	JIAN LIU
FECHA SOLICITUD:	07 DE ENERO DE 2001

ASUNTO: CONTESTACIÓN A REQUERIMIENTO OFICIAL

DILIA RODRIGUEZ D´ALEMAN, en mi condición de apoderada de la sociedad solicitante, por el presente escrito doy respuesta a su auto notificado por estado de 26 DE ENERO DE 2006 y vence 26 DE ABRIL DE 2006 y me permito presentar los siguientes documentos:

- Shen, YC, Chen CY, Kuo YH. "A new diterpenoid from Taxus mairei". J. Nat. Prod. 26 jun. 1998; 61 (6): 838-40.

- Zamir LO, Nedea ME, Zhou ZH, Caron G, Sauriol F, Mamer O. "Isolation and semisynthesis of a bioactive taxane from Taxus Canadensis". Phytochemistry. Feb. 1996; 41 (3): 803-5.

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- Rao KV. "Taxol and related taxones I. Taxones of Taxus brevifolia bark". Pharm. Res. Ab. 1993; 10 (4): 521-4. Univ. Of Florida.

- Ojika M, Kigoshi H, Kuyama H, Niwa H, Yamada K. "Studies on Pteridium aquilinum var. Latiusculum IV. Isolation of threel p-hydroxystyrene glycosides and an efficient method for the isolation of ptaquiloside an unstable bracken carcinogen. J. Nat. Prod. Jul-Ag. 1985; Vol. 48, N° 4:634-636.

- Wang Feng-Peng and Pelletier S. W. "Diterpenoid alkaloids from Aconitum crassicaule". J. Nat. Prod. Ene-Feb 1987; Vol. 50, N° 1: 55-62.

Dado que mediante este memorial se cumple con los requerimientos realizado por ese Despacho en virtud del artículo 46 de la Decisión 486 de la Comisión de la Comunidad Andina, agradezco continuar con el presente trámite.

Señor Jefe,

DILIA RODRIGUEZ D´ALEMAN
MRO/srch

A New Taxane Diterpenoid from *Taxus mairei*

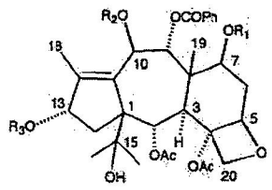
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Received December 26, 1997

A new 11(15→1)-abeotaxane diterpene, taxumairol K (**1**), has been isolated from the ethanolic extract of the roots of Formosan *Taxus mairei* (Lemee & Levl.) S. Y. Hu. The structure of **1** was determined as 9α-(benzoyloxy)-2α,4α-diacetoxy-5β,20-epoxy-1β,7β,10β,13α-tetrahydroxy-11(15→1)-abeotaxane on the basis of spectral analysis. Taxumairol K (**1**) exhibited mild cytotoxicity against HeLa tumor cells.

The discovery of paclitaxel as a clinically useful antitumor agent has stimulated great interest in searching for practical and renewable sources of paclitaxel and other taxoids.[1−8] *Taxus mairei* (Lemee & Levl.) S. Y. Hu (Taxaceae) is an evergreen shrub distributed over mountainous regions of the northern and central parts of Taiwan. The roots of *T. mairei* have been used in Chinese folk medicine for thetreatment of diabetes.[9] Previous studies on the diterpenoids in the roots of *T. mairei* have resulted in the isolation of taxumairols A−F together with many known taxoids.[10−13] Taxumairols A−F have a 6/8/6-membered ring skeleton. Continued investigation of *T. mairei* has resulted in the identification of a novel abeotaxane with a 5/7/6-membered ring system from the roots of this species. Herein, we wish to report the isolation, structure elucidation, and cytotoxicity of taxumairol K (**1**).



1 $R_1 = R_2 = R_3 = H$
2 $R_1 = R_2 = R_3 = Ac$

Extensive chromatography of the ethanolic extract of *T. mairei* roots on Sephadex LH-20, Si gel, and RP-C18 gave a residue, which was finally purified by HPLC to furnish taxumairol K (**1**). Taxumairol K (**1**), [α] −8.5° (MeOH), had the composition $C_{31}H_{40}O_{11}$ as derived by a combination of EIMS and DEPT spectroscopy. Its UV and IR bands indicated the presence of benzoyl (229 nm), hydroxyl (3475, 3444, 3421 cm^{-1}), and acetyl (1722 cm^{-1}) groups. This was also supported by the base peak at *m/z* 105 due to the benzoyl ion and peaks at *m/z* 447 and *m/z* 405 arising from loss of benzoic acid, 2 mol of H_2O, and acetic acid from the molecular ion. The presence of one benzoyl and two acetyls as well as four hydroxyls was verified from the ^1H and ^{13}C NMR spectral data of **1** (Table 1). A taxene skeleton was inferred from the observation of characteristic resonances, such as four methyl singlets [δ 1.02 (17-Me), 1.23 (16-Me), 1.74 (19-Me), and 1.80 (18-Me)] and the corresponding ^{13}C NMR signals.[1,2] The ^1H NMR spectrum of **1** consisted of several overlapped, broad lines, and spectral analysis was difficult.[11] Fortunately, the relationship between each proton such as H-2/H-3, H-6a/H-6b/H-7, and H-9/H-10 in **1** could be observed from the COSY spectrum. In accord with this, the overlapped H-2 (δ 5.97) and H-9 (δ 5.98) signals were distinguished by their correlations with H-3 (δ 3.19) and H-10 (δ 4.70), respectively. The H-7 signal was assigned at δ 4.47 because it correlated with signals at δ 1.75/2.60 (H-6). Although correlations between H-13/H-14a and H-13/H-14b could not be observed, cross-peaks between H-14a/H-14b appeared at δ 1.58 and δ 2.20. The remaining H-20 signals (δ 4.44/4.46) were overlapped with those of H-13 and H-7. Detailed analysis of the ^1H and ^{13}C NMR spectra revealed that compound **1** is an isomer of an 11(15→1)-abeotaxane,[14,15] namely, 7,9-dideacetyltaxayuntin, isolated from *T. yunnanensis*.[16] These isomeric compounds had similar ^1H and ^{13}C NMR spectra. Detailed comparison of the ^1H and ^{13}C NMR spectrum of **1** with those of taxayuntin F further suggested that the benzoate was at C-9 and the acetates at C-2 and C-4. This was further backed by the observation that the chemical shift difference between the diastereotopic C-20 protons was small, as expected for the presence of a C-2 acetate, while a C-2 benzoate would have led to a larger difference.[14,17−22] An HMBC study of **1** failed to correlate the signals between H-10 and C-12 and between H-9 and benzoyl carbon via long-range coupling due to low sensitivity.

Upon acetylation, compound **1** yielded a pentaacetate **2** ($C_{37}H_{46}O_{14}$), which is an isomer of 10,13-diacetyltaxayuntin E.[23] Compound **2** showed the H-7 signals at δ 5.55, H-10 at δ 6.44, and H-13 at δ 5.62 in its ^1H NMR spectrum, indicating that two of the three hydroxyls occur at the C-7 and C-13 positions in **1**. The remaining hydroxyl group was assigned to either the C-9 or the C-10 position. To confirm the position of the benzoyloxy group and to assign the ^1H and ^{13}C NMR signals of **2**, COSY, HMQC, and HMBC experiments were performed. The connectivities between H-10 (δ 6.44) and C-12 (δ 147.1) and between H-9 (δ 6.32) and

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Table 1. ^1H and ^{13}C NMR Spectral Data of Taxumairol K (1)

position	^{13}C	carbon type[a]	^1H[b]	COSY	HMBC
1	66.6	s			H-2
2	70.2	d	5.97 (overlap)	H-3	H-3
3	43.7	d	3.19 (brs)	H-2	H-2
4	79.7	s			H-20
5	85.4	d	4.92 (d, 6.6)		
6	34.7	t	2.60 (m) 1.75 (m)	H-7	
7	70.2	d	4.47 (m)	H-6	H-5[c]
8	43.4	s			Me-19
9	80.8	d	5.98 (overlap)	H-10	
10	68.7	d	4.70 (brs)	H-9	
11	137.9	s			Me-18[c]
12	146.2	s			H-10,[c] Me-18[c]
13	77.6	d	4.49 (m)		Me-18[c]
14	39.5	t	2.20 (m, β), 1.58 (m)		
15	76.6	s			Me-16, Me-17
16	25.6	q	1.23 s		Me-17[c]
17	27.5	q	1.02 s		Me-16[c]
18	11.3	q	1.80 s		
19	14.0	q	1.74 s		
20A	75.1	t	4.44 (overlap)	H-20	
20B			4.46 (overlap)	H-20	
OAc	170.4	s			
	21.7	q	2.00 s		
OAc	171.3	s			
	22.4	q	2.16 s		
$OCOC_6H_5$	167.7	s			H-9,[c] o-C_6H_5
i	130.4	s			
o	129.8	d	8.00(d, 6.0)	*m*-C_6H_5	*p*-C_6H_5, *m*-C_6H_5
m	128.3	d	7.43 (m)	*o*,*p*-C_6H_5	
p	133.0	d	7.53 (m)	*m*-C_6H_5	*o*-C_6H_5
OH-7			3.44 (brs)	H-7	
OH-10			3.85 (brs)	H-10	
OH-13			1.50 (overlap)	H-13	

[a] s = C, d = CH, t = CH_2, q = CH_3. Multiplicities and assignments made by the DEPT and HMBC. [b] Multiplicities and coupling constants in Hz in parentheses. [c] Data taken from taxumairol K pentaacetate (2).

the benzoyl carbonyl (δ 167.5) unambiguously established the benzoyl moiety at the C-9 position. The stereochemistry of **1** and **2** was tentatively determined by comparison of their coupling constants in the ^1H NMR spectra and chemical shifts of ^{13}C NMR spectra with those of known compounds.

Biological study on tumor cytotoxicity revealed that taxumairol K (**1**) showed weak activity toward cervical carcinoma (HeLa) with an IC_{50} value of 9.2 μg/mL. Taxumairol K (**1**) was inactive against hepatoma (Hep-3B) tumor cells (IC_{50} 40 μg/mL). Compound **1** is a new compound having the rearranged 11(15→1)-abeotaxane skeleton. The broad and poorly resolved NMR spectra might be explained by the existence of slow interconversion of two conformations (twist-chair and twist-boat) of **1**. The first occurrence of the 5/7/6-membered structure in *T. mairei* is of significance from a biogenetic point of view.

Experimental Section

General Experimental Procedures. Optical rotations were recorded on a JASCO DIP-1000 polarimeter. IR and UV spectra were measured on Hitachi T-2001 and Hitachi V-3210 spectrophotometers, respectively. EIMS and FABMS were recorded on a VG Quattro 5022 mass spectrometer. The ^1H and ^{13}C NMR and DEPT spectra were recorded on a Varian FT-300 instrument and HMQC and HMBC spectra on a FT-400 AMX spectrometer.

Plant Material. The roots of *T. mairei* (Lemee & Levl.) S. Y. Hu were purchased in Kaohsiung, Taiwan, in October 1993. A voucher specimen has been deposited at the Institute of Marine Resources, National Sun Yat-sen University.

Extraction and Isolation. Dried roots (60 kg) were extracted as previously described.[12] A portion (1.5 kg) was obtained from the $CHCl_3$/MeOH-soluble fraction. Part of the residue (200 g) was applied to a Sephadex LH-20 column (1 kg) and eluted with MeOH to afford a taxoid-containing fraction (85 g). The taxoid fraction was chromatographed on a silica gel column (850 g) and eluted with a solvent mixture of $CHCl_3$–Me_2CO by increasing the polarity to provide seven fractions. Fraction F (9 g) was applied to a LH-20 column and eluted with MeOH to give a residue (2.83 g). Rechromatography of the residue on a silica gel column and elution with *n*-Hex/$CHCl_3$/MeOH according to the following ratios and volumes (7:7:1, 6:6:1, 5:5:1, 4:4:1, 3:3:1, 2:2:1, and 1:1:1 each 1 L) yielded four fractions, fractions 1 (0.42 g), 2 (1.07 g), 3 (0.16 g), and 4 (0.61 g). Separation of fraction 2 (1.07 g) on a reversed phase C18 column (35 g) using solvent mixtures of MeOH/H_2O of decreasing polarity (7:3, 7:4, 7:5, 7:6, and 1:1, each 300 mL) afforded five fractions; fractions A (taxumairol B, 18 mg), B (taxumairol C, 7 mg), C (taxumairol E, 250 mg), D (baccatin III, 30 mg), and E (65 mg). Purification of fraction E by HPLC (silica gel) using *n*-hexane–$CHCl_3$–MeOH (5:5:1) as solvent system yielded taxumairol K (**1**, 35 mg).

Taxumairol K (1): amorphous solid; $[\alpha]^{25}_D$ −8.5° (*c* 0.65, MeOH); UV (MeOH) λ_{max} (log ϵ) 229 (4.22) nm; IR (neat) ν_{max} 3475, 3444, 3421, 2981, 1722, 1369, 1238

cm^{-1}; ^{1}H and ^{13}C NMR ($CDCl_3$) spectral data are listed in Table 1; EIMS (70 eV) *m/z* 465 (0.1), 447 (2), 405 (1), 345 (1), 297 (11), 183 (5), 123 (9), 105 (100).

Acetylation of taxumairol K (1). Acetylation (Ac_2O–Pyr 1:1; room temperature) of **1** (5 mg) gave a solid (6 mg) after workup: $[\alpha]^{25}_D$ −33° (*c* 0.25, MeOH); UV (MeOH) λ_{max} (log ϵ) 229 (4.17) nm; IR (neat) ν_{max} 3451, 2952, 1735, 1436, 1234 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.16 (1H, d, J = 7.6 Hz, H-2), 2.98 (1H, d, J = 6.7 Hz, H-3), 4.97 (1H, d, J = 7.6 Hz, H-5), 1.80 (1H, m, H-6a), 2.66 (1H, m, H-6b), 5.54 (1H, t, J = 7.1 Hz, H-7), 6.32 (1H, d, J = 10.8 Hz, H-9), 6.43 (1H, d, J = 10.8 Hz, H-10), 5.62 (1H, t, J = 6.9 Hz, H-13), 1.70 (1H, m, H-14a), 2.28 (1H, m, H-14b), 1.19 (3H, s, H-16), 1.16 (3H, s, H-17), 1.87 (3H, s, H-18), 1.76 (3H, s, H-19), 4.50 (1H, d, J = 7.3 Hz, H-20a), 4.41 (1H, d, J = 7.3 Hz, H-20b), 2.21, 2.01, 1.82, 1.64 (3H × 5, *s*, $OCOCH_3$); ^{13}C NMR (75.4 MHz, $CDCl_3$) δ 67.6 (s, C-1), 68.2 (d, C-2), 44.0 (d, C-3), 79.0 (s, C-4), 85.0 (d, C-5), 34.7 (t, C-6), 70.5 (d, C-7), 43.7 (s, C-8), 78.7 (d, C-9), 67.9 (d, C-10), 136.0 (s, C-11), 147.1 (s, C-12), 78.7 (d, C-13), 46.9 (t, C-14), 75.5 (s, C-15), 25.0 (q, C-16), 27.5 (q, C-17), 11.7 (q, C-18), 13.3 (q, C-19), 74.7 (t, C-20), 168.0, 169.0, 170.0, 170.2, 170.6 (s, $OCOCH_3$), 129.6 (s, *i*-C_6H_5), 129.9 (d, *o*-C_6H_5), 128.5 (d, *m*-C_6H_5), 133.4 (d, *p*-C_6H_5), 167.5 (s, $OCOC_6H_5$), 22.7, 21.9, 21.6, 21.0, 20.6 (q, $OCOCH_3$); EIMS (70 eV) *m/z* 595 (0.1), 537 (2), 476 (1), 432 (1), 372 (3), 354 (1), 339 (6), 330 (1), 312 (2), 297 (3), 252 (7), 180 (2), 105 (100), 77 (23), 59 (41), 43 (73); FABMS *m/z* 737 $[M + Na]^+$, 715 $[M + H]^+$, 655 $[M - OAc]^+$; HRFABMS *m/z* $[M + H]^+$ 715.2956 ($C_{37}H_{47}O_{14}$ requires 715.2966).

Cytotoxicity Assay. A bioassay against HeLa (cervical carcinoma) and Hep-3B (hepatoma) tumor cells was based on reported procedures.[24–26] The cells for assay were cultured in RPMI-1640 medium supplemented with a 5% CO_2 incubator at 37 °C. The cytotoxicity assay depends on the binding of methylene blue to fixed monolayers of cells at pH 8.5, washing the monolayer, and releasing the dye by lowering the pH value. In summary, samples and control standard drugs were prepared at a concentration of 1, 10, 40, and 100 μg/mL. After seeding 2880 cells/well in a 96-well microplate for 3 h, 20 mL of sample or standard agent was placed in each well and incubated at 37 °C for 3 days. After the medium was removed from the microplates, the cells were fixed with 10% formal saline for 30 min and then dyed with 1% (w/v) methylene blue in 0.01 M borate–buffer (100 μL/well) for 30 min. The 96-well plate was dipped into a 0.01 M borate–buffer solution four times in order to remove the dye. Then, 100 μL/well ethanol–0.1M HCl (1/1) was added as a dye-eluting solvent, and the absorbance was measured on a microtiter plate reader (Dynatech, MR 7000) at a wavelength of 650 nm. The IC_{50} value was defined by comparison with the untreated cells as the concentration of test sample resulting in 50% reduction of absorbance. Mytomycin C and actinomycin D were used as standard compounds, which both exhibited IC_{50} values of 0.01 μg/mL under the above conditions.

Acknowledgment. We thank Ms. Chao-lein Ho and Ms. Shiu-ching Yu of the NSC Southern NMR and MS Instrument Center at National Sun Yat-sen University and Ms. Ru-ron Wu, Department of Chemistry, National Cheng Kung University, for measurements of NMR and MS spectral data. The authors thank the National Science Council and the National Institute of Health, Republic of China, for financial support (Grant Nos. NSC86-2732-B110-002 and DOH 87-HR-509).

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ISOLATION AND SEMI-SYNTHESIS OF A BIOACTIVE TAXANE FROM *TAXUS CANADENSIS*

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(*Received in revised form* 21 *June* 1995)

Key Word Index—Taxanes; *Taxus canadensis*; Taxaceae; taxcultine.

Abstract—A minor bioactive taxane, isolated from the needles of *Taxus canadensis* and semi-synthesized, was shown to be taxcultine.

INTRODUCTION

Taxus canadensis, a small ramping bush abundant in Quebec, seems to differ from other yews by the content of its taxanes. 9-Dihydro-13-acetylbaccatin III is found in amounts three to seven times the content of taxol [1]. Various derivatives of 9-dihydro-13-acetylbaccatin III and an uncyclized taxane (canadensene) were also found in the needles of the canadian yew [2,3]. This paper reports the isolation of taxcultine (1) from *T. canadensis* needles and its semi-synthesis. This taxane was previously obtained from cell cultures of *T. baccata* (hence the given name of taxcultine) [4,5].

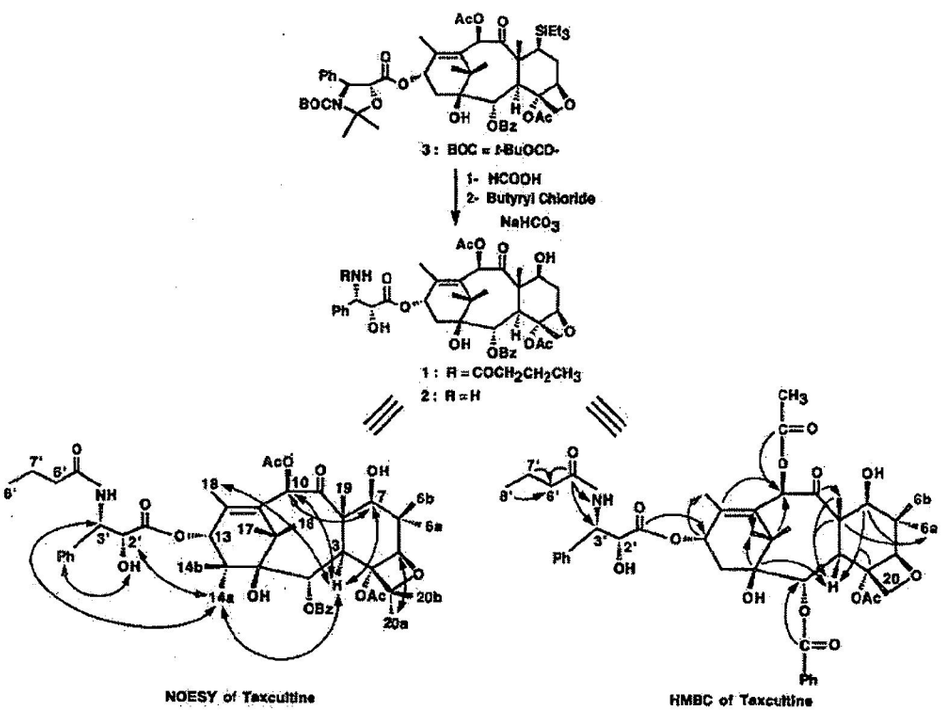
RESULTS AND DISCUSSION

Compound 1 was a minor metabolite (0.0008% yield from *T. canadensis* needles) which was found in the same flash chromatography fraction as taxol, 9-dihydro-13-acetylbaccatin III and numerous minor taxanes. Semi-preparative HPLC using a reversed phase column and a gradient solvent system of water–acetonitrile allowed the isolation of a mixture containing 1 and 10-hydroxyacetylbaccatin VI, a new taxane recently identified [3]. The final purification of 1 was achieved by analytical HPLC.

A detailed high resolution NMR analysis (500 MHz ^{1}H NMR, HMQC and HMBC) allowed the assignments of the protons and the carbons. The two-dimensional ^{1}H–^{13}C correlations (HMBC) obtained for 1 are shown on the lower right side of the figure: the arrows indicate carbon–hydrogen connectivities with the arrow head indicating location of the proton. The NOESY experiment enabled us to confirm the stereochemistry of the diterpenoid core skeleton and that the 'U-shape' of taxol is retained in 1 as can be seen by the long range correlation between methyl-18 and H-3. The α-side correlations are shown on the lower left side of the figure. Absolute confirmation of the structure of 1 was obtained by semi-synthesis. 10-Deacetylbaccatin III isolated from *T. canadensis* needles was protected [6] and coupled with *N,O*-protected β-phenylisoserine to give 3 and hydrolysed [7] to give 2. Acylation with butyryl chloride yielded a compound, which, after purification (53% yield), was identical to 1. High resolution NMR and mass spectra of the semi-synthetic and naturally occurring 1 were identical. In addition, HPLC chromatograms of the natural and semi-synthetic products under three different mobile phase conditions were identical. Compound 1 promotes the assembly of microtubules, and its activity is slightly higher than that of taxol, but lower than that of taxotere. The semi-synthesis can now provide large quantities of 1 for further testing.

EXPERIMENTAL

General. NMR: 500 MHz (^{1}H), and 125 MHz (^{13}C) in $CDCl_3$. Low resolution Xe fast atom bombardment mass spectra were obtained in glycerol. The samples were dissolved in DMSO before addition of 0.5 ml glycerol. High resolution mass spectra were made similarly in glycerol–DMSO, at a resolving power of 12 000. Semi-prep. HPLC was performed on a Waters Delta Prep 3000 instrument coupled with a Model 481 variable wavelength detector with two ODS-2 MAG-9 reversed phase semi-prep. column (Whatman) connected in series (9.4 × 500 mm), at 3 ml min^{-1}. Detection was at 227 nm. For all semi-prep. HPLC sepns, a gradient of acetonitrile (25–100%) in H_2O over 50 min was used unless otherwise stated. The analyt. HPLC system was a Perkin–Elmer instrument equipped with



an isocratic and binary LC pump (Model 250). The sepns were achieved on two ODS-2 reversed phase columns (Whatman) connected in series (4.6 × 300 mm), at 1 ml min^{-1}.

Plant material. Taxus canadensis was collected in Quebec, during the autumn season. The plants were stored at 4° in sterilized sand and peatmoss and were dried before grinding.

Extraction, isolation and purification. The extraction procedure is the same as described in ref. [3]. Semi-prep. HPLC allowed isolation of a peak (R_t = 38 min) which contained a mixt. of **1** and 10-hydroxyacetylbaccatin VI [3]. The final purification of **1** was achieved by analyt. HPLC (isocratic conditions, eluent H_2O–MeCN (29:21; R_t = 49.2 min) and yielded 4 mg **1** as a powder, UV λ_{max} MeOH 230 nm (ε12800). FAB-MS *m/z*: 820.35424, $C_{44}H_{54}NO_{14}$ requires 820.35443. ^{1}H NMR: δ 0.90 (3H, *t*, *J* = 7.3 Hz, H-8'), 1.15 (3H, *s*, H-17), 1.26 (3H, *s*, H-16), 1.62 (2H, *m*, H-7'), 1.68 (3H, *s*, H-19), 1.82 (3H, *s*, H-18), 1.88 (1H, *dd*, *J* = 10.7, 15.6 Hz, H_b-6), 2.18 (2H, *t*, *J* = 7.3 Hz, H-6'), 2.25 (3H, *s*, Ac-10), 2.28–2.32 (2H, *m*, H-14), 2.34 (3H, *s*, Ac-4), 2.46 (1H, *br s*, OH-2'), 2.54 (1H, *ddd*, *J* = 15.6, 9.3, 6.5 Hz, H_a-6), 3.79 (1H, *d*, *J* = 7.0 Hz, H-3), 4.19 (1H, *d*, *J* = 8.3 Hz, H_b-20), 4.30 (1H, *d*, *J* = 8.3 Hz, H_a-20), 4.40 (1H, *dd*, *J* = 10.7, 6.3 Hz, H-7), 4.68 (1H, *br s*, H-2'), 4.94 (1H, *d*, *J* = 9.3 Hz, H-5), 5.57 (1H, *dd*, *J* = 9.3, 2.4 Hz, H-3'), 5.67 (1H, *d*, *J* = 7.0 Hz, H-2), 6.20 (1H, *d*, *J* = 9.3 Hz, NH-4'), 6.21 (1H, *m*, H-13), 6.28 (1H, *s*, H-10), 7.35–7.40 (5H, *m*, CHPh), 7.51–8.11 (5H, *m*, COPh). ^{13}C NMR: δ 9.16 (C-19), 13.53 (C-8'), 14.70 (C-18), 18.79 (C-7'), 20.54 (Ac-10), 21.70 (C-17), 22.28 (Ac-4), 26.66 (C-16), 35.41 (C-6), 35.41 (C-14), 38.33 (C-6'), 43.02 (C-15), 45.33 (C-3), 54.37 (C-3'), 58.39 (C-8), 72.16 (C-7), 72.16 (C-13), 73.04 (C-2'), 74.79 (C-2), 75.37 (C-10), 76.25 (C-20), 78.90 (C-1), 81.03 (C-4), 84.12 (C-5), 127.00, 128.17, 129.04, 137.84 (CHPh), 128.75, 130.21, 133.71, 137.84, 166.88 (COPh), 133.14 (C-11), 142.11 (C-12), 170.30 (Ac-4), 171.16 (Ac-10), 172.86 (C-1'), 172.86 (C-5'), 203.62 (C-9).

Biological activity: in vitro *polymerization.* A modification of the method in ref. [8] was used to measure the ability of taxol, **1** and taxotere to stimulate the assembly of tubulin into microtubules. Microtubule protein was isolated from fresh bovine brain by two assembly–disassembly cycles similar to the method of ref. [9], but with some modifications [10]. Initial rates and final values of absorbance at 350 nm were for taxotere: 0.149 AU min^{-1}; 0.56 AU; **1**: 0.068 AU min^{-1}; 0.40 AU; taxol: 0.058 AU min^{-1}; 0.28 AU; control: 0.003 AU min^{-1}; 0.06 AU.

Semi-synthesis: the coupling compound (**3**). The side chain of **3** was prepd according to ref. [7]; FAB-MS of the methyl ester *m/z*: 336.1812, $C_{18}H_{26}NO_5$ requires 336.1811; FAB-MS of the acid-CH_3 *m/z*: 306.1341, $C_{16}H_{20}NO_5$ requires 306.1342. The coupling procedure of ref. [6] was used. After work up, the product was purified by semi-prep. HPLC (R_t = 63.1 min). The product (**3**) was obtained as a solid (85% yield). Starting material (10%; R_t = 50.8 min) was recovered. Compound **3**: FAB-MS *m/z* $[MH]^+$ 1004, $[MH - AcOH]^+$ 944.

^{1}H NMR: (two rotamers present; second downfield signals represented by an asterisk; integrations of split signals are half indicated value) δ 0.56 (6H, *m*, $SiCH_2$), 0.91 (9H, *m*, $SiCH_2CH_3$), 1.55 (9H, *s*, *t*-Bu), 1.80 (1H, *m*, H_a-6), 2.48 (1H, *m*, H_b-6), 2.14 (2H, *m*, H-14), 3.74 (1H, *d*, $J = 7.0$ Hz, H-3), 3.77 (*1H, *d*, $J = 7.1$ Hz, H-3), 4.09 (1H, *d*, $J = 8.3$ Hz, H_b-20), 4.10 (*1H, *d*, $J = 8.1$ Hz, H_a-20), 4.23 (1H, *d*, $J = 8.0$ Hz, H_a-20), 4.24 (*1H, *d*, $J = 8.3$ Hz, H_b-20), 4.46 (1H, *m*, H-7), 4.86 (1H, *d*, $J = 7.3$ Hz, H-5), 4.87 (*1H, *d*, $J = 8.0$ Hz, H-5), 5.06 (1H, *br s*, H-2'), 5.55 (1H, *br s*, H-3'), 5.64 (1H, *d*, $J = 7.1$ Hz, H-2), 5.65 (*1H, *d*, $J = 6.9$ Hz, H-2), 6.24 (1H, *t*, $J = 8.8$ Hz, H-13), 6.43 (1H, *s*, H-10), 6.44 (*1H, *s*, H-10), 7.3–7.4 (5H, *m*, CHPh), 7.50–8.04 (5H, *m*, COPh).

Semi-synthetic taxcultine (**1**). The coupling compound (**3**) (4.0 mg, 0.0040 mmol) was stirred in 0.3 ml 95–97% HCO_2H at room temp. The reaction was monitored by TLC and after 4 h, HCO_2H was removed by a flow of N_2. After drying under vacuum, the residue was dissolved in EtOAc (0.5 ml), and $NaHCO_3$ (10 mg) and butyryl chloride (5 μl, 0.047 mmol) were added. After stirring for 1 h, EtOAc (10 ml) was added to the soln, which was washed with brine and dried over $MgSO_4$. After filtration and evapn, the residue was purified by semi-prep. HPLC ($R_t = 31.7$ min). Compound **1** was obtained as a solid [1.8 mg, 53% yield based on compound **3**, $R_f = 0.25$, EtOAc–hexane (7:3)]; FAB-MS *m/z*: 820.35423, $C_{44}H_{54}NO_{14}$ requires 820.35443. NMR values were identical to that of the naturally occurring **1**. Co-elution of the semi-synthetic and naturally occurring samples of **1** on analyt. HPLC showed identical R_t values in 3 different conditions: 1—linear gradient of AcCN (25–100%) in H_2O over 50 min, $R_t = 31.7$ min; 2—linear gradient of AcCN (25–100%) in H_2O over 140 min, $R_t = 57.1$ min; 3—isocratic conditions with AcCN (43%) in H_2O, $R_t = 45.9$ min.

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Taxol and Related Taxanes. I. Taxanes of *Taxus brevifolia* Bark

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The published procedures for the isolation of taxol from the Pacific yew (*Taxus brevifolia*) and other species of *Taxus* are cumbersome, and the yields of taxol are in the range of 0.0075–0.01%. This paper describes a simple and efficient procedure for the isolation of taxol and its major natural analogues from the bark of *T. brevifolia* consisting of a single chromatographic column (using silica gel, Florisil, or a reverse-phase C18-silica), followed by crystallization. Isolated yields of taxol from five "pooled" bark samples (blended from many different batches by the supplier) were in the range of 0.02–0.04%, and from bark collected from a more restricted locale, yields reached 0.06%. The procedure also yielded taxol analogues, such as 10-deacetylbaccatin III (0.02–0.04%), 10-deacetyltaxol-7-xyloside (0.06–0.1%), taxol-7-xyloside (0.005–0.01%), 10-deacetyltaxol (0.01–0.02%), 10-deacetylcephalomannine-7-xyloside (0.006–0.01%), and cephalomannine (0.005–0.007%). Of these, 10-deacetyltaxol-7-xyloside is the most abundant taxane in the Pacific yew bark.

KEY WORDS: *Taxus brevifolia;* taxol; taxane-7-xylosides; 10-deacetylbaccatin III; 10-deacetyltaxol; cephalomannine.

INTRODUCTION

Taxol (1; Fig. 1a) is a diterpene ester isolated from the bark of *Taxus brevifolia* by Wani *et al.* in 1971 (1). Its unique mechanism of action (2) and the promising results in clinical trials in ovarian (and possibly breast) carcinomas (3,4) have made it currently the most important new antitumor agent under test and procurement of taxol has assumed a high priority.

The bark of *T. brevifolia* is currently considered to be the best source of taxol, although taxol occurs widely in various parts of several species of *Taxus;* however, the needles of one of these species may become the preferred source (5–7). Semisynthesis from natural taxanes (8,9) and total synthesis are also being actively pursued.

While the bark of *T. brevifolia* will serve as the major source of taxol for the short term, no publications on the isolation of taxanes from the bark of *T. brevifolia* have appeared since 1986. Two previous papers (10,11) described the isolation of 10-deacetylbaccatin III, 7-epi-taxol, 10-deacetyl-10-oxotaxol, and decinnamoyl taxinine J in low yields (0.003%) (from side fractions of a large-scale taxol isolation (of the bark) process. Miller *et al.* (12,13) obtained taxol (0.0108%), cephalomannine (0.0163%), and 10-deacetyltaxol from *T. wallichiana*. Senilh *et al.* (14) isolated, from the bark of *T. baccata*, taxol (0.0165%), cephalomannine (0.0064%), 10-deacetyltaxol (0.0029%), 10-deacetyl cephalomannine (0.0034%), taxol-7-xyloside (0.0058%), cephalomannine-7-xyloside (0.0039%), 10-deacetyltaxol-7-xyloside (0.022%), and 10-deacetylcephalomannine-7-xyloside (0.0096%). Isolation of taxol/taxanes required multiple chromatographic columns, countercurrent distribution, and preparative thin-layer chromatography (tlc). A large-scale taxol process from the bark of *T. brevifolia* was developed by Polysciences Laboratories Inc., but only sketchy details were published (11), with a reported yield of 0.0075–0.01%.

The present work was begun with the objective of developing a simpler and more efficient procedure for the isolation of taxol and its natural analogues from the bark of *T. brevifolia*. Results described here indicate that a simplified procedure consisting essentially of one chromatographic column, followed by direct crystallization of the major components, is possible. Since the steps involved are fewer, there is the possibility of realizing higher yields.

MATERIALS AND METHODS

General. Melting points were determined on a Fisher-Johns apparatus and are uncorrected. The following instruments were used to record the spectra described here: uv, Perkin-Elmer, Lambda 3B; ir, Perkin-Elmer, PE-1420; and NMR, Nicolet NY-300. Thin-layer chromatography (tlc) was performed with silica gel (Merck H60-P254/366), using acetone and/or methanol in chloroform. Visualization was by uv and/or spraying with 1 *N* H_2SO_4, followed by charring.

Plant Material. The plant materials (bark) supplied as *Taxus brevifolia* were purchased or obtained from a variety of sources. Two samples of bark collected from a limited area (habitat) were purchased from Mr. Charles Edson, Research Resources Inc., Medford, OR 97501, during May–September 1990, and are designated E-1 and E-2. One other similarly collected (restricted-area) sample (PB-1) was received from Pacific Biotechnology Inc., 17627 N.E. 65th, Redmond, WA 98052. During September 1990 to September 1991, three pooled samples, designated NC-1, NC-2, and NC-3 (pooled from many collections and similar to what is being used in the large-scale isolation), were received from the National Cancer Institute. Another such pooled sample (MM) was received from Medimolecule Inc., 4620 Starboard Drive, Boulder, CO 80301. During January 1992, still another pooled batch (WP) was purchased from Mr. Walt Phillips, 12108 S.E. Boise, Portland, OR 97226. All of the bark samples were reduced to <0.5-cm mesh, air-dried at ambient temperature before extraction.

Extraction Method. Scheme I shows the extraction and purification steps used in the isolation. The ground bark (3 kg) was immersed in methanol for 24 hr at ~23°C and drained. The process was repeated two or three more times, based on the uv absorbance (275 nm) as an indicator of extraction. The combined extract was concentrated (<45°C) to a syrup (~500 mL), diluted with water (to 1.5 L), and partitioned with chloroform (3×; 1, 0.8, and 0.8 L). Each of the organic layers was washed once with water and concentrated to a syrup, which was then dried *in vacuo*. The solid, designated the "chloroform-extract solids" (approx 60 g) was kept in a tightly stoppered bottle until use.

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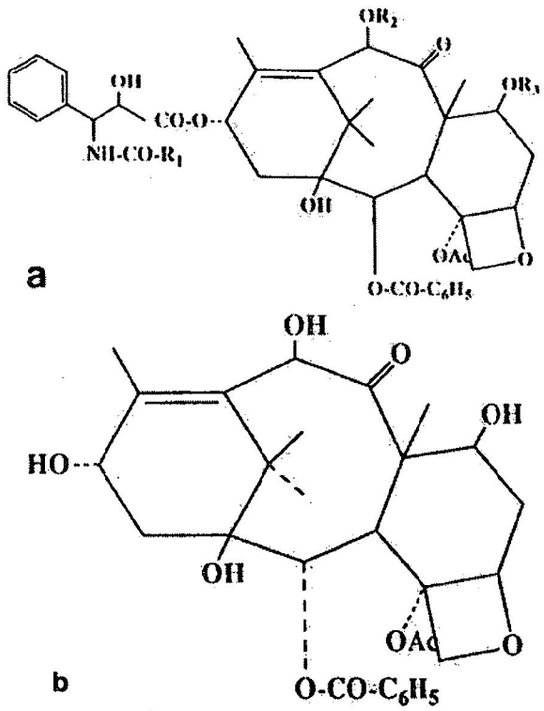


Fig. 1. (a) (1) Taxol: R_1 = benzoyl; R_2 = acetyl; R_3 = H. (2) Cephalomannine: R_1 = tiglyl; R_2 = acetyl; R_3 = H. (4) 10-Deacetyltaxol-7-xyloside: R_1 = benzoyl; R_2 = H; R_3 = D-xylosyl. (5) 10-Deacetylcephalomannine-7-xyloside: R_1 = tiglyl; R_2 = H; R_3 = D-xylosyl. (6) Taxol-7-xyloside: R_1 = benzoyl; R_2 = acetyl; R_3 = D-xylosyl. (7) 10-Deacetyltaxol: R_1 = benzoyl; R_2 = H; R_3 = H. (b) (3) 10-Deacetylbaccatin III.

Column Chromatography. This was performed using three adsorbents: Si gel (Fisher A-743-1, 230–425 mesh), Florisil (Fisher F 101-3), and a reverse phase-type adsorbent such as C8- or C18-bonded silica (Lichroprep, 15- to 25-μm size; E. M. Science). Possible variations from batch to batch of these adsorbents must be recognized and corrected for.

High-performance liquid chromatography (HPLC) was carried out using Waters analytical/semiprep system, consisting of one each of Nos. 510 and 6000A pumps, a No. 680 gradient controller, a No. U6K injector, a No. 440 uv detector set at 254 nm, and a Fisher Recordall 5000 recorder. Standard analytical columns (4.6 × 250 mm) containing silica gel or C8-, C18-, or CN-bonded silica gel columns were purchased from Fisher. Semiprep columns were prepared using 1-in.-diameter stainless-steel columns (Waters) or glass columns (Ace Glass Co.) and packing with the various materials listed above. In general, samples were applied as either solutions or fine suspensions at a concentration of approximately 10%. The columns were run either by gravity flow or with pressure (10–100 psi) as needed.

To a column prepared from the reverse-phase adsorbent (C18-silica, 15 to 35-μm size, 100–125 g) in 25% acetonitrile/water was applied the chloroform extract solid (10 g) in the same solvent (100 mL), and the column developed using a step gradient of acetonitrile in water (in increments of 5 until 50%, then in increments of 10%) until a concentration of 80% acetonitrile was reached. The change of solvent was based on the monitoring data, i.e., when the concentration of the substances being eluted became low, which happened generally within 500–1000 mL of the eluant. Fractions (10–20 mL) were collected and monitored by tlc, analytical HPLC, and uv absorption (275 nm). Based on these data, the appropriate fractions were combined and concentrated. The three major components, 10-deacetylbaccatin III, 10-deacetyltaxol-7-xyloside, and taxol, appeared in that order, being eluted, respectively, with 25–30, 30–40, and 40–50% acetonitrile/water. Individual components were obtained by concentration of the appropriate fractions and crystallization from acetone/ligroin or acetonitrile/water.

Alternatively, to a silica column prepared from 100 g of the adsorbent in chloroform/ligroin (1:1), the sample (chloroform-extract solids, 10 g) in the same solvent (100 MI) was added. The column was developed with the same solvent, which was changed by step gradient to chloroform, 2 and 5% acetone in chloroform, and subsequently 2, 5, and 10% methanol in chloroform. Collection of fractions and monitoring were carried out as described above. Generally, the three major compounds—taxol, 10-deacetylbaccatin III, and 10-deacetyltaxol-7-xyloside—emerged in this order, in the chloroform/acetone and the chloroform/methanol eluates. The solids were crystallized using acetone/ligroin or acetonitrile/water.

The same procedure was used with Florisil instead of silica gel, following the same elution sequence.

Scheme I

Methanol extract (4×) of the bark of *Taxus brevifolia*

↓ Concentration, partition between water and chloroform

Water | Chloroform

Chloroform → Concentration → Concentrate solids → Chromatography → Fractions → Crystallization

RESULTS

The chloroform-extractable solids represented approximately 2% of the weight of the bark. Of the taxanes found in the extract, the following were recognized and isolated (for structures, see Figs. 1a and b): taxol (1), cephalomannine (2), 10-deacetylbaccatin III (3), 10-deacetyltaxol-7-xyloside (4), 10-deacetylcephalomannine-7-xyloside (5), taxol-7-xyloside (6), and 10-deacetyltaxol (7). The last four compounds are isolated from the bark of *T. brevifolia* for the first time, although these were originally reported from the bark of *T. baccata* (14), and cephalomannine and 10-deacetyltaxol from *T. wallichiana* (12,13).

The isolated yields of taxol and two of its major analogues from the various batches of the bark are shown in

Table I. Yields of Taxol and its Analogues from the Bark of *T. brevifolia*

Source	Quality	Method	Percentage[a]		
			Taxol	A	B
1. ED-1	3.8 kg	Silica	0.04	0.03	0.08
2. ED-2	1 kg	C-18	0.06	0.03	0.1
3. ED-2	1 lb	Florisil	0.05	0.03	0.08
4. PB-1	100 g	C-18	0.06		
5. NC-1	1 kg	C-18	0.02	0.03	0.06
6. NC-2	1 kg	C-18	0.04		
7. NC-3	1 lb	C-18	0.03	0.03	0.055
8. MM	1 lb	C-18	0.02		
9. WP	1 kg	C-18	0.03	0.03	0.07

[a] A, 10-deactylbaccatin III; B, 10-deacetyltaxol-7-xyloside.

Table I. Since these studies were carried out using eight batches of the bark, the overall yields quoted here represent the actual range. The yield data were mostly obtained from the reverse-phase column technique, followed by direct crystallization. In one or two comparative runs, the silica column was found to give comparable yields.

Taxol and its analogues described here were also tested in an L-1210 cell culture assay using the protocols described by Thayer *et al.* (15) and the results are shown in Table II.

In the following section, the physical and spectral properties of the various taxanes are described. The ir and ^{13}C-NMR data were obtained and are available upon request.

Taxol (1). Taxol was crystallized first from acetonitrile/water and then from acetone/hexane to form colorless needles, m.p. 219–222°C with decomposition [lit., 213–216°C (1)]; [α], two batches: −51.2, −51.2 (methanol) [lit., −49, methanol (1)], −23.8, −24.0 (pyridine) [lit., −21, pyridine (14)]; and $E_{228\ nm}$, 30,300, compared to that of a reference sample of pure taxol, 30,200. The proton and ^{13}C-NMR spectra were identical to those recorded from an authentic sample of taxol obtained from the NCI.

Analytical HPLC (6), RP-CN, 42% acetonitrile in water, and monitored at 254 nm, showed a single peak (Fig. 2). The chromatogram was further analyzed in detail at both 254 and 220 nm using a photodiode array detector and NEC 386/33i computer. Peak purity determination showed that the taxol peak was 99.2% homogeneous. The area of the taxol peak accounted for essentially 100% of the total area of all the peaks when seen at 254 nm and 98.8% of the total peak area as seen at 220 nm. Two of the samples were submitted for elemental analysis. *Anal.* Calcd for $C_{47}H_{51}NO_{14}$: C, 66.10; H, 6.02; N, 1.64. Found (two batches): C, 66.00, 66.21; H, 6.04, 6.09; N, 1.60, 1.63.

Table II. Cytotoxic Activity of Taxol and its Analogues

Compound	IC_{50} (mEq/ml)
1. Taxol	0.02
2. Cephalomannine	0.04
3. 10-Deacetyltaxol-7-xyloside	0.6
4. 10-Deacetylcephalomannine-7-xyloside	0.8
5. Taxol-7-xyloside	0.07
6. 10-Deacetyltaxol	0.02
7. 10-deacetylbaccatin III	>10

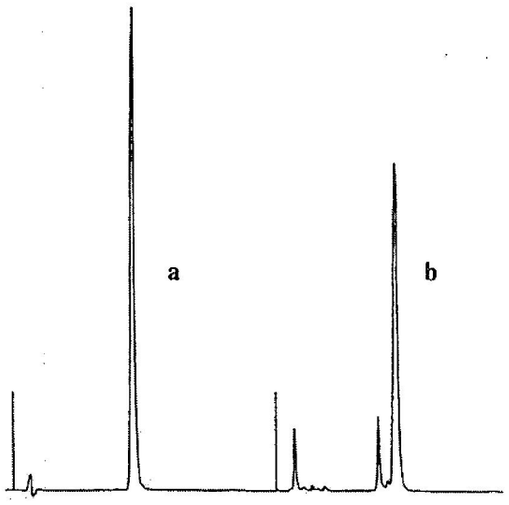


Fig. 2. HPLC profile of (a) taxol and (b) cephalomannine/taxol mixture.

10-Deacetylbaccatin III (3). The compound crystallized as colorless glistening plates, m.p. 232–234°C [lit., 234–236°C (12)]; [α], −40.7 (methanol). The proton NMR spectrum was identical to that recorded earlier (8). *Anal.* Calcd for $C_{29}H_{36}O_{10}$: C, 63.96; H, 6.59. Found: C, 63.82; H, 6.59.

10-Deacetyltaxol-7-xyloside (4). This crystallized from acetone or methanol/water as colorless needles, m.p. 245–248°C; [α], −1 (pyridine), −14 (methanol) [lit. (14), 246–248°C and −2 (pyridine)]. Its ^1H-NMR was identical to that recorded earlier (14). *Anal.* Calcd for $C_{50}H_{57}NO_{17}$, H_2O: C, 62.42; H, 6.18; N, 1.46. Found: 62.66, 62.64; H, 6.24, 6.29; N, 1.58, 1.54.

Acetylation of the compound (acetic anhydride/pyridine, 100°C, 30 min) gave an acetate crystallized from ether, m.p. 216–218°C [lit., 219–220°C (14)]. The ^1H-NMR spectrum was identical to that described (14). *Anal.* Calc. for $C_{60}H_{67}NO_{22}$, H_2O: C, 61.47; H, 5.93. Found: C, 61.35; H, 5.93.

10-Deacetylcephalomannine-7-xyloside (5). Crystallization from acetone gave colorless needles, yield 0.004–006%, m.p. 245–249°C [lit., 250–252°C (14)]. The ^1H-NMR spectrum was identical to that recorded earlier (14). *Anal.* Calcd for $C_{48}H_{59}NO_{17}$, H_2O: C, 61.35; H, 6.54; N, 1.49. Found: 61.54, 61.62; H, 6.84, 6.80; N, 1.42, 1.44.

Taxol-7-xyloside (6). Crystallization from acetone/hexane gave colorless plates, yield 0.004–0.006%, m.p. 233–237°C [lit., 236–238°C (14)]. The ^1H-NMR spectrum was identical to that recorded earlier (14). *Anal.* Calcd for $C_{52}H_{59}NO_{18}$: C, 63.34; H, 6.03; N, 1.42. Found: C, 63.08; H, 6.11; N, 1.42.

10-Deacetyltaxol (7). This crystallized for the first time from aqueous acetonitrile as colorless needles, yield 0.01–0.02%, m.p. 192–196°C. The ^1H-NMR spectrum was identical to that recorded earlier (13,14). *Anal.* Calcd. for $C_{45}H_{49}NO_{13}$: C, 66.57; H, 6.08; N, 1.73. Found: C, 66.42; H, 6.14; N, 1.78.

Cephalomannine (2). Crystallization from acetone/ligroin gave colorless silky needles, yield 0.005–0.007%, m.p. 185–188°C [lit., 186–188°C (12)], identical to an authentic sample of cephalomannine by HPLC and ^{1}H-NMR spectra (12,14).

DISCUSSION

A simple and practical scheme suitable for the isolation of taxol and several of its important natural analogues is described here, consisting of a single chromatographic column followed by crystallization(s), starting from the "chloroform extract solids." In contrast, the methods described in the literature for taxol and related taxanes (10–14), including the large-scale process developed by Polysciences Inc. (11), consist of multiple column and various other manipulative steps. For the operation of the column described here, three adsorbents were studied, and all three were found to give comparable yields of the key components, such as taxol, 10-deacetylbaccatin III, and 10-deacetyltaxol-7-xyloside. The reverse-phase column is more convenient, and most compounds listed here can be purified by direct crystallization. When this does not happen with the minor components because of their low content, the combined fractions and the mother liquors from the crystallizations may be rechromatographed on a silica column for successful crystallization.

Bark samples collected from narrow habitat gave isolated yields of taxol in the range of 0.04–0.06%. On the other hand, the yields of taxol from five "pooled" samples of the bark, which are comparable to those being used in the large-scale isolation of taxol, ranged from 0.02 to 0.04%, in comparison to the generally reported yield of 0.01%.

The purity of the taxol was determined by a number of criteria. Elemental analysis, molecular extinction coefficient, optical rotation, and proton and ^{13}C-NMR spectra all indicated excellent purity. HPLC analysis showed a purity range of 98.5–99% for the taxol samples (Fig. 2). Finally, the cytotoxic IC_{50} value in the L-1210 cell culture assay was in the same range as that of an authentic sample of taxol.

The isolation yield of taxol from the bark of *T. brevifolia* is generally accepted as 0.01% (dry wt basis). The yield of taxol on large-scale extraction (Polysciences Inc.) is quoted as 0.004–0.016% (11). In a congressional hearing (16) conducted by Rep. Ron Weiden (Oregon), it was stated that the yields of taxol by the Hauser process have been improved from 0.0067 to 0.01% (20,000 lb being needed, instead of 30,000 lb per kg of taxol). This number (0.01%) is extensively quoted as a standard in both the scientific and the lay press. This paper documents a substantially improved isolation procedure.

Taxol may also be prepared by semisynthesis from 10-deacetyl baccatin III (8,9), which is present to the extent of 0.01–0.1% in the needles of *T. baccata* and others (6,8,17) and is being isolated on a large scale in France. However, since large amounts of the bark are being processed in the United States for taxol, it would be prudent to isolate this important analogue also at the same time. The procedure outlined here makes this possible, and the bark is shown to be a good source for this compound.

ACKNOWLEDGMENTS

The author expresses appreciation to the late Dr. Richard Donovick of the National Cancer Institute (NCI) for getting our laboratory involved in taxol research and to Dr. Gordon M. Cragg of the NCI for the reference samples of taxol and cephalomannine. Appreciation is also expressed to Mr. Richard M. Davies for the extractions, the cell culture data, and the spectra described here. The author also thanks Mr. Frank Farland and Mr. David Lee of Waters Associates for the HPLC analysis of the taxol samples.

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STUDIES ON *PTERIDIUM AQUILINUM* VAR. *LATIUSCULUM*, IV.[1] ISOLATION OF THREE *p*-HYDROXYSTYRENE GLYCOSIDES AND AN EFFICIENT METHOD FOR THE ISOLATION OF PTAQUILOSIDE, AN UNSTABLE BRACKEN CARCINOGEN

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Bracken, *Pteridium aquilinum* Kuhn var. *latiusculum* Underw. (Pteridaceae), has been shown to be carcinogenic to various experimental animals (1-3). Recently we have performed fractionation of the aqueous extract of bracken by means of the assay based on carcinogenicity and isolated a new, unstable, norsesquiterpene glucoside, ptaquiloside (**1**) from the carcinogenic fraction (4,5). Then, the carcinogenicity of ptaquiloside (**1**) has been proved (6). Subsequent examination of this carcinogenic fraction led to the isolation of two *p*-hydroxystyrene glycosides, ptelatoside-A (**2**) and ptelatoside-B (**3**) (7). The original procedure for isolating ptaquiloside (**1**) was, however, inefficient and complicated and led to only ca. 0.02% yield based on the dry weight of bracken (4-6).

We report here the development of an efficient and convenient method for the isolation of the bracken carcinogen (**1**) in a yield five times greater than the original procedure (4-6). In the course of the isolation of **1** by the new procedure, three *p*-hydroxystyrene glycosides, *p*-hydroxystyrene β-D-glucoside (**4**) (8), Glycoside A (**5**) (9), and a new compound ptelatoside-C (**6**) were isolated in addition to ptelatoside-A (**2**) and ptelatoside-B (**3**).

In our new procedure, the dried, powdered bracken was extracted with H_2O at room temperature and the aqueous extract was treated with the resin Amberlite XAD-2. The resin adsorbate was eluted with MeOH. The aqueous solution of the MeOH eluate residue was extracted with *n*-BuOH repeatedly and the combined *n*-BuOH extracts were chromatographed on silica gel with EtOAc, EtOAc-MeOH (92:8), and MeOH, successively. Separation of the EtOAc-MeOH (92:8) eluate by reverse-phase column chromatography followed by reverse-phase hplc afforded ptaquiloside (**1**). The fractions eluted with MeOH were separated by chromatography first on alumina and then on silica gel and subsequently by reverse-phase hplc to give Glycoside A (**5**) and ptelatoside-C (**6**) together with ptelatoside-A (**2**) and ptelatoside-B (**3**). *p*-Hydroxystyrene β-D-glucoside (**4**) was obtained by chromatography of the *n*-BuOH extracts (see above) on silica gel with $CHCl_3$-MeOH (9:1) and subsequent reverse-phase hplc.

The molecular formula $C_{19}H_{26}O_{10}$ of ptelatoside-C (**6**) was determined from the molecular ion peak in fabms and the ^{13}C-nmr spectrum, and by consideration of the components [the aglycone derivative (**8**) and two sugar components]. The presence of the *p*-*O*-substituted styrene moiety in **6** was deduced by the spectral data: ^1H nmr δ 7.06 and 7.38 (4H, m, AA'BB' type); ^{13}C nmr δ 157.1 (s), 133.4 (s), 128.5 (d, 2×C), 117.8 (d, 2×C); uv (see Experimental section). Acidic methanolysis of ptelatoside-C (**6**) gave a mixture of methyl glycosides of D-glucose and L-arabinose together with the phenol (**8**) (7), a MeOH adduct of the aglycone, *p*-hydroxystyrene. Acetylation of **6** gave the corresponding hexaacetate (**7**). In the ^{13}C-nmr spectrum of **6**, the signals at δ 68.7 (t, C-6'), 100.9 (d, $^1J_{CH}$=164

[1]The article by Ojika *et al.* (7) can be considered as Part III of the series.

Hz, C-1′), and 104.0 (d, $^1J_{CH}$=164 Hz, C-1″) revealed the sugar moiety of **6** to be represented as α-L-arabinopyranosyl-(1→6)-β-D-glucopyranosyl, which was further supported by the detailed analysis of the ^{1}H-nmr spectrum of the hexaacetate (**7**). Thus, the structure of ptelatoside-C was determined to be **6**.

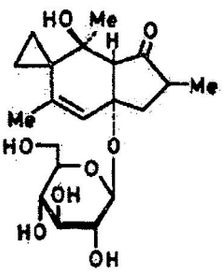
So far, reports on the natural occurrence of *p*-hydroxystyrene and its glycosides are rare. While *p*-hydroxystyrene and its β-D-glucoside were isolated from *Papaver somniferum* (10) in 1945 and *Cheilanthes kuhnii* (8) in 1980, respectively, isolation of four *p*-hydroxystyrene glycosides from *Dicranopteris dichotoma* (9), *Microlepia obtusiloba* (9), and *P. aquilinum* var. *latiusculum* (7) was only recently described.

EXPERIMENTAL

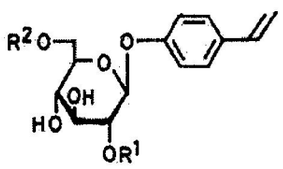
GENERAL EXPERIMENTAL PROCEDURES.—The ir and uv spectra were recorded on a JASCO Model IRS spectrophotometer and a JASCO UVIDEC-510 spectrophotometer, respectively. ^{1}H-nmr and ^{13}C-nmr spectra were measured at 90 MHz and 22.5 MHz, respectively, with a JEOL JNM-FX90QE spectrometer. The ^{1}H-nmr spectrum at 270 MHz was obtained on a JEOL JNM-GX270 spectrometer. Mass spectra were recorded on a Hitachi RMU-6C spectrometer and a JEOL JMS-DX300 instrument. Optical rotations were measured with a JASCO DIP-181 digital polarimeter. For column chromatography, Fuji-Davison silica gel BW-820MH, Fuji-Davison 5D ODS (ODS-silica gel), and Merck neutral alumina (Aluminiumoxid 90, Activity II-III) were used. Preparative tlc was conducted on Merck silica gel PF_{254}. Hplc was performed with a JASCO TRI ROTAR-II apparatus using a reverse-phase column of Develosil ODS-5 (250×10 mm id).

PLANT MATERIAL.—Plants of *P. aquilinum* var. *latiusculum* were collected in the Nayoro area of Hokkaido, Japan, in July 1983, and were identified by botanist H. Wakita. A voucher specimen, KY-N-83, has been deposited at the Herbarium of the Laboratory of Organic Chemistry, Department of Chemistry, Nagoya University.

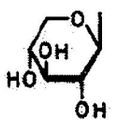
EXTRACTION PROCEDURE AND PREPARATION OF THE *n*-BuOH EXTRACT.—The dried, finely powdered, plant materials (3.0 kg) were stirred in H_2O (30 liters) at room temperature for 2 h and filtered. After the resin Amberlite XAD-2 (wet volume, 12 liters) was added to the filtrate, the mixture was stirred at room temperature for 1 h and filtered. The filtrate was again treated with fresh Amberlite XAD-2 (wet volume, 12 liters). MeOH (30 liters) was added to the combined



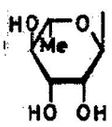
1



2 R^1=H, R^2=



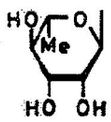
3 R^1=

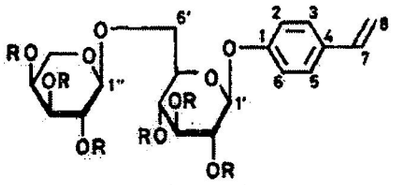


, R^2=H

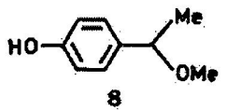
4 R^1=H, R^2=H

5 R^1=H, R^2=





6 R=H
7 R=Ac



8

resin XAD-2 and the mixture was stirred for 3 h at room temperature. The mixture was filtered and the resin was washed with MeOH (30 liters). The combined MeOH solution was concentrated under reduced pressure, and the residue (90 g) was dissolved in H_2O saturated with *n*-BuOH (1 liter). The aqueous solution was extracted with *n*-BuOH saturated with H_2O (5×500 ml). Concentration of the combined organic solution under reduced pressure afforded the *n*-BuOH extract as a dark-brown, amorphous solid (33 g). By the procedure described above, another 1.2 g of the *n*-BuOH extract was prepared from 100 g of the dried, finely powdered, plant materials.

ISOLATION OF PTAQUILOSIDE (**1**), GLYCOSIDE A (**5**), AND PTELATOSIDE-C (**6**).—The *n*-BuOH extract (33 g) (see above) was chromatographed on silica gel (600 g) with EtOAc, EtOAc-MeOH (92:8), and MeOH, successively. Concentration of the fractions eluted with EtOAc-MeOH (92:8) under reduced pressure gave a residue (9 g), which was chromatographed on ODS-silica gel (180 g) with H_2O-MeOH (60:40). The resulting crude **1** was further purified by hplc with H_2O-MeOH (50:50) and freeze-dried to give pure **1** (3 g, 0.1%) as a colorless amorphous powder. Concentration of the fractions eluted with MeOH under reduced pressure afforded a residue (18 g), which was chromatographed on alumina (300 g) with MeOH and MeOH-H_2O (9:1, 8:2, 7:3). Evaporation of the fractions eluted with MeOH-H_2O (9:1, 8:2, 7:3) under reduced pressure yielded a residue (2.4 g), which was chromatographed over silica gel (70 g) with $CHCl_3$-MeOH (4:1). Further separation by hplc with EtOH-H_2O (25:75) afforded Glycoside A (**5**) (28 mg, 0.0009%, amorphous powder), $[\alpha]^{22}D$ −66.5° (c 1.00, H_2O), Lit. $[\alpha]^{20}D$ −65.5° (c 2.55, MeOH) (9), and ptelatoside-C (**6**) (62 mg, 0.002%, amorphous powder) together with ptelatoside-A (**2**) (135 mg, 0.005%) (7) and ptelatoside-B (**3**) (200 mg, 0.007%) (7). Glycoside A (**5**) was identified by comparison (uv, ^1H nmr, ^{13}C nmr) with an authentic sample. Ptelatoside-C (**6**): $[\alpha]^{22}D$ −67.3° (c 0.797, H_2O); uv λ max (MeOH) 254 (ε 15800), 288 (shoulder, 1800), 298 (shoulder, 1100) nm; ir ν max (KBr) 3420, 1628, 1605, 1510, 1170 cm^{-1}; ^1H nmr (90 MHz, CD_3OD) δ 5.11 (1H, dd, J=11.0, 1.1 Hz, H-8), 5.64 (1H, dd, J=17.6, 1.1 Hz, H-8), 6.68 (1H, dd, J=17.6, 11.0 Hz, H-7), 7.06 and 7.38 (4H, m, AA'BB' type, H-2, H-3, H-5, H-6); ^{13}C nmr (22.5 MHz, D_2O) δ 66.9 (t, C-5″), 68.7 (t, C-6′), 69.0 (d), 70.2 (d), 71.6 (d), 73.1 (d), 73.7 (d), 76.3 (d), 100.9 (d, $^1J_{CH}$=164 Hz, C-1′), 104.0 (d, $^1J_{CH}$=164 Hz, C-1″), 114.1 (t, C-8), 117.8 (d, C-2, C-6), 128.5 (d, C-3, C-5), 133.4 (s, C-4), 136.7 (d, C-7), 157.1 (s, C-1); fabms *m/z* 437 $(M+Na)^+$.

ISOLATION OF *p*-HYDROXYSTYRENE β-D-GLUCOSIDE (**4**).—The *n*-BuOH extract (1.2 g) (see above) was chromatographed on silica gel (55 g) using $CHCl_3$-MeOH (9:1, 4:1, 3:1). Evaporation of the fractions eluted with $CHCl_3$-MeOH (9:1) afforded a residue (136 mg), which was further separated by hplc with MeOH-H_2O (45:55) to give **4** (28 mg, 0.028%), mp 193-194° (H_2O), $[\alpha]^{17}D$ −62.3° (c 0.278, MeOH). Compound **4** was identified by comparison (uv, ir, and ^1H nmr) with an authentic sample, mp 193-194° (H_2O), $[\alpha]^{11}D$ −69.4° (c 0.274, MeOH), synthesized by the known procedure (11).

METHANOLYSIS OF PTELATOSIDE-C (**6**).—A mixture of **6** (107 mg) in MeOH (6 ml) containing H_2SO_4 (0.09 ml) was refluxed for 4 h and then neutralized by addition of $NaHCO_3$. Concentration of the mixture under reduced pressure afforded a residue, which was diluted with H_2O (10 ml) and extracted with $CHCl_3$ (3×10 ml). Concentration of the aqueous layer under reduced pressure gave a residue (335 mg), which was separated by chromatography first on silica gel (10 g) with EtOAc-MeOH (20:1) and then on silica gel (4 g) with EtOAc-MeOH (40:1) and finally by preparative tlc with EtOAc-MeOH (15:4). There was obtained a mixture of anomeric methyl L-arabinosides (18 mg) and of anomeric methyl D-glucosides (51 mg), the former being identified as methyl 2,3,4-tri-*O*-acetyl-β-L-arabinopyranoside (mp 75.5-76.0°) and the latter as methyl 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranoside (mp 64.5-65.0°) after acetylation and purification [preparative tlc, CCl_4-Me_2CO (5:1)]. Concentration of the $CHCl_3$ extracts under reduced pressure afforded a residue (52 mg), separation of which by chromatography on silica gel (5 g) with $CHCl_3$-EtOAc (10:1) gave the phenol (**8**) (18 mg), mp 102.0-102.5°, which was proved to be identical with the authentic sample by spectral (uv, ir, and ^1H nmr) comparison.

PTELATOSIDE-C HEXAACETATE (**7**).—A mixture of **6** (2 mg) in Ac_2O (0.5 ml) and anhydrous pyridine (0.5 ml) was stirred at room temperature for 14 h and concentrated under reduced pressure. The residue was purified by preparative tlc with $CHCl_3$-EtOAc (2:1) to afford **7** (2.2 mg) as an amorphous powder; ^1H nmr (270 MHz, C_6D_6) δ 1.61 (6H, s, 2×Ac), 1.64, 1.71, 1.72, 1.76 (3H each, s each, 4×Ac), 2.83 (1H, dd, J=12.9, 1.7 Hz, H-5″), 3.50 (2H, m, H-5′, H-6′), 3.62 (1H, dd, J=12.9, 3.0 Hz, H-5″), 3.87 (1H, brd, J=8.9 Hz, H-6′), 4.23 (1H, d, J=7.3 Hz, H-1″), 4.83 (1H, d, J=7.9 Hz, H-1′), 5.06 (2H, m, H-3″, H-4′), 5.08 (1H, dd, J=10.9, 1.0 Hz, H-8), 5.17 (1H, m, H-4″), 5.40 (1H, dd, J=9.9, 9.2 Hz, H-3′), 5.53 (1H, dd, J=9.9, 7.9 Hz, H-2′), 5.58 (1H, dd, J=9.6, 7.3 Hz, H-2″), 5.66 (1H, dd, J=17.5, 1.0 Hz, H-8),

6.65 (1H, dd, J=17.5, 10.9 Hz, H-7), 6.99 and 7.38 (4H, m, AA'BB' type, H-2, H-3, H-5, H-6).

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DITERPENOID ALKALOIDS FROM *ACONITUM CRASSICAULE*[1]

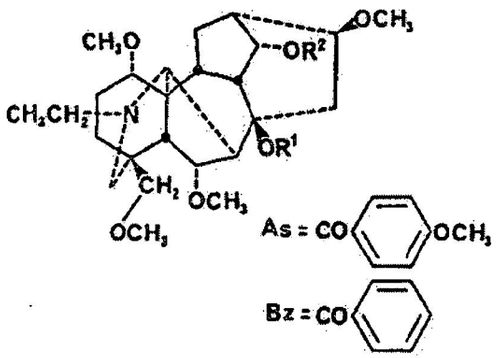
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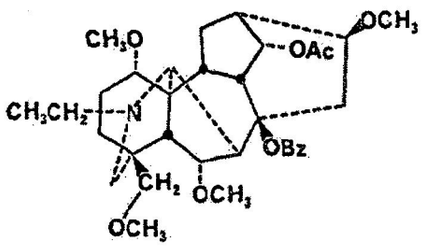
ABSTRACT.—From the roots of *Aconitum crassicaule* three new and five known diterpenoid alkaloids have been isolated. The new alkaloids are crassicaudine [**1**], crassicausine [**6**], and crassicautine [**11**]. The known alkaloids are chasmanine [**3**], crassicauline A [**5**], foresaconitine [**2**], forestine [**9**], and yunaconitine [**10**]. The isolation and determination of the structures of these alkaloids are described. The structures of the new alkaloids were established on the basis of spectral data and correlation with compounds of established structures. The structure of crassicaudine [**1**] was established by synthesis from chasmanine [**3**]. The structure of forestine [**9**] has been confirmed by correlation with crassicauline A [**5**].

The Chinese plant *Aconitum crassicaule* W.T. Wang (Ranunculaceae) has been shown to contain several diterpenoid alkaloids (1-3). In the course of studies of the total alkaloids of freshly collected roots of this plant, three new alkaloids—crassicaudine [**1**], crassicausine [**6**], crassicautine [**11**]—and five known alkaloids—chasmanine [**3**], crassicauline A [**5**], foresaconitine [**2**], forestine [**9**], and yunaconitine [**10**]—have been isolated. In this paper, we wish to describe the isolation and structure determination of these compounds. The total alkaloids obtained by an ion-exchange resin method (4) were separated by a combination of column chromatography, preparative tlc, and centrifugal tlc (Chromatotron®).

Crassicaudine [**1**] crystallized from Et_2O as colorless needles, mp 148-150°; $[\alpha]^{27}D$ +15° (*c* 0.32, $CHCl_3$); $C_{34}H_{47}NO_8$ (based on hrms); ^1H nmr δ1.06 (3H, t, J=7 Hz, NCH_2CH_3), 1.34 (3H, s, $OCOCH_3$), 3.16, 3.36 (each 3H, s, 2× OCH_3), 3.26 (6H, s, 2× OCH_3), 3.66 (2*H*, AB type, J=12 Hz, 18-Hz), 4.10 (1H, dd, J_1=6 Hz, J_2=1 Hz, 6 β-*H*, 5.10 (1H, t, J=4.5 Hz, 14 β-*H*, 7.36-8.20 (5H, m, aromatic protons); ms *m/z* 597(M^+), 566(M-OCH_3), 506(M-OCH_3-HAc), 105(100). The ^{13}C-nmr data for crassicaudine [**1**] are given in Table 1. The ^1H- and ^{13}C-nmr spectral data of crassicaudine are very similar to those of foresaconitine [**2**] (5,6) except for differences of



1 R^1=Ac, R^2=Bz (crassicaudine)
2 R^1=Ac, R^2=As (foresaconitine)
3 R^1=R^2=H (chasmanine)
3a R^1=H, R^2=Bz (14-benzoylchasmanine)



4

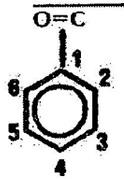
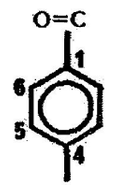
[1]This article commemorates the 50th year of publication of the *Journal of Natural Products* (formerly *Lloydia*).

[2]Member of the Editorial Advisory Board of the *Journal of Natural Products* (*Lloydia*) since 1980.

TABLE 1. Carbon-13 Chemical Shifts and Assignments for Crassicaudine [1], Foresacontine [2] (5), Crassicauline A [5] (20), Crassicausine [6], Aminoalcohol of Crassicausine [7], Bikhaconine [8], Forestine [9] (15), Yunaconitine [10] (11), Crassicautine [11], and Pseudaconine [12] (21, 22)

Carbon	Compounds									
	10	5	1	2	6	7	8	9	11	12
1	83.2	84.8	85.1	85.1	85.3	85.4	85.6	85.4	83.1	84.5
2	33.7	25.9	26.4	26.4	26.3	26.0	26.0	26.0	33.4	33.8 (35.9)[a]
3	71.3	35.4	35.0	34.9	34.8	34.9	34.9	34.9	71.8	72.2
4	43.2	39.1	39.1	39.1	39.1	39.2	39.3	39.3	43.2	43.5
5	47.4	48.9	49.3	49.2	49.0	49.1	49.9	49.6	47.5	49.3
6	82.3	83.3	82.3	82.6	82.9	82.8	82.3	82.5	82.6	82.4
7	44.8	50.4	44.9	44.9	48.4	48.2	52.4	48.3 (49.2)[a]	48.4	52.2
8	85.6	85.3	85.9	85.9	78.4	78.4	72.7	73.7	78.6	72.9
9	48.8	40.9	49.3	49.3	46.5	49.5	50.4	53.6	46.1	50.4
10	40.8	44.9	44.1	43.9	36.3	35.2	42.2	42.3	35.6	42.0
11	50.3	49.8	50.4	50.3	50.6	50.4	50.1	50.2	50.8	50.2
12	35.3	34.5	29.0	29.0	36.5	36.3	36.1	36.4	36.7	35.9 (33.8)[a]
13	74.8	75.1	39.1	39.1	85.4	76.4	76.7	76.1	75.3	76.9
14	78.6	78.9	75.6	75.4	80.3	80.2	80.6	80.1	79.1	79.6
15	39.6	38.9	37.9	37.9	41.6	41.7	39.8	41.9	41.5	40.1
16	83.6	83.9	83.5	83.5	84.0	84.0	84.5	83.3	83.9	83.2
17	61.6	61.6	61.6	61.7	61.4	62.0	62.5	62.2	61.1	62.4
18	76.8	80.4	80.5	80.4	80.3	80.2	79.5	80.6	79.1	77.5
19	48.8	53.8	53.9	53.8	53.9	53.6	53.9	53.6	47.6	48.7
N-CH_2	47.4	49.1	48.9	49.0	48.6	48.6	49.3	49.2 (48.3)[a]	48.6	47.4
CH_3	13.3	13.1	13.4	13.4	13.5	13.5	13.5	13.6	13.3	13.7
1′	56.8	56.1 (55.6)[a]	56.5	56.6	56.2	56.1	56.1	56.3	55.8	56.3
6′	58.8	57.8 (56.2)[a]	57.8	57.8	58.6	58.4	57.3	58.3	58.6	57.4
8′	—	—	—	—	58.8	58.8	—	—	58.8	—
16′	57.8	58.7 (59.3)[a]	55.9	56.0	58.8	58.8	57.7	57.5	58.8	57.9
18′	59.1	59.1 (57.8)[a]	59.0	59.1	59.0	59.0	59.1	59.2	59.1	59.4
O=C(8′)	169.9	169.8	169.6	169.8	—	—	—	—	—	—
CH_3	21.7	21.3	21.7	21.8	—	—	—	—	—	—

TABLE 1. *Continued*

Carbon	Compounds									
	10	**5**	**1**	**2**	**6**	**7**	**8**	**9**	**11**	**12**
 O=C	—	—	166.3	—	—	—	—	—	—	—
1	—	—	130.7	—	—	—	—	—	—	—
2,6	—	—	128.4	—	—	—	—	—	—	—
3,5	—	—	129.7	—	—	—	—	—	—	—
4	—	—	132.8	—	—	—	—	—	—	—
 O=C	166.1	166.3	—	166.2	166.5	—	—	166.6	166.3	—
1	122.6	123.1	—	123.0	123.3	—	—	122.4	123.3	—
2,6	131.7	131.9	—	131.8	131.8	—	—	131.8	131.8	—
3,5	113.8	114.1	—	113.7	113.5	—	—	113.8	113.5	—
4	163.5	163.8	—	163.5	163.5	—	—	163.4	163.3	—
OCH_3	55.4	55.4 (59.7)[a]	—	55.4	55.4	—	—	55.4	55.3	—

[a]Numbers in parentheses are old values.

signals in the aromatic region. Comparison of aromatic signals of foresaconitine with those of crassicaudine indicated that crassicaudine possesses a benzoyl group instead of an anisoyl group as in foresaconitine. Hydrolysis of crassicaudine with 10% NaOH in MeOH gave chasmanine [3]. The results lead to **1** or **4** as the structure for crassicaudine. Alkaloids without an oxygen substituent at C(13), but bearing a C(8)-OAc and a C(14)-OBz or C(14)-OAs, show a 3H singlet for the acetate methyl group between δ 1.34-1.46 and a 1H signal for the C(14)-H between δ 5.00-5.11. Examples are 8-acetyl-14-benzoylneoline (7), foresaconitine (5,6), isodelphinine (8), penduline (9,10), vilmorrianine A (6), and 3-acetylvilmorrianine A (6). However, alkaloids with the reverse arrangement, viz. C(8)-OBz or C(8)-anisoyl and C(14) OAc, such as ezochasmaconitine (11) and anisoylchasmaconitine (11,12), show a 3H singlet between δ 1.76-1.78 and a 1H signal for the C(14)-H between δ 4.80-4.82. Because crassicaudine shows a 3H singlet at δ 1.34 and a 1H signal at δ 5.10, clearly it has structure **1**. This structure was confirmed by synthesis from chasmanine [3]. Benzoylation of 3 with benzoyl chloride and pyridine gave 14-benzoylchasmanine [3a]. Acetylation of the latter with Ac_2O and *p*-toluenesulfonic acid gave 8-acetyl-14-benzoylchasmanine, mp 154-156°, that was identical in all respects with an authentic sample of crassicaudine [1]. Incidentally, both crassicaudine and foresaconitine can be separated by preparative tlc over alumina but not over silica gel GF254.

Crassicausine [6] was isolated as an amorphous compound showing one spot on tlc (alumina, Et_2O-1% MeOH); $[\alpha]^{23}D$ +32.7° (*c* 0.20, $CHCl_3$); $C_{34}H_{49}NO_9$ (based on hrms); ir 3500(OH), 1715(ester), 1609, 770(aromatic); ^1H nmr δ 1.10 (3H, t, J=7 Hz, NCH_2CH_3), 1.5-1.8 (2H, m, 3-H_2), 2.99, 3.26, 3.54 (each 3H, s, 3× OCH_3), 3.29 (6H, s, 2×OCH_3), 3.84 (3H, s, aromatic methoxyl), 4.00 (1H, dd, J_1=6 Hz, J_2=1 Hz, 6β-*H*), 4.87 (1H, d, J=4.5 Hz, 14β-*H*), 6.90, 8.04 (4H, A_2B_2 type, -OCO-C_6H_4-); ^{13}C nmr data for crassicausine are given in Table 1; ms *m/z* 615 (M^+), 600($M-CH_3$), 584($M-OCH_3$, 77.7%), 554(10.2), 466(23.9), 432(13.2), 149(12.8), 135(*p*-CO-C_6H_5-OCH_3, 100).

Of the aliphatic methoxyls, the signal at δ 2.99 is at a particularly high field. The one oxygen to be accounted for in addition to those of the anisoyl and five methoxyl groups may be present as a hydroxyl group based on ir absorption at 3500 cm^{-1}. The mass spectrum exhibited an intense characteristic fragment at *m/z* 584 (77.7%) corresponding to the loss of a methoxyl on C(1) (13,14).

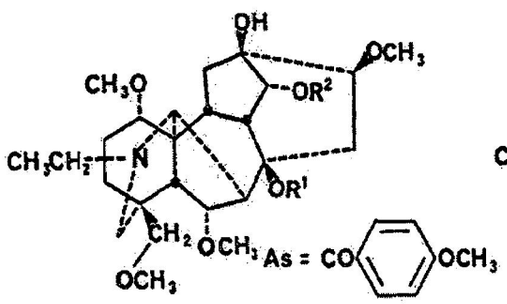
The ^{13}C-nmr data (Table 1) of crassicausine are similar to those of forestine [9] (15), which suggested the presence of methoxyls at C(1), C(6), C(16), and C(18). The ^{13}C-nmr spectrum of crassicausine showed the presence of two quaternary carbon atoms bearing an oxygenated group; one signal at 78.4 ppm has been assigned to C(8) and the other at 85.4 ppm must be either C(9) or C(13) because the C(14)β-proton is a doublet. The presence of a hydroxyl group at C(13) is suggested by the fact that crassicausine does not manifest any signal downfield of 28 ppm apart from the one at 26.3 ppm attributed to the C(2) methylene. If there were no OH group at C(13), one would expect the C(12) methylene triplet to appear around 26-30 ppm. Therefore, structure **6** may be assigned to crassicausine. This assignment was established by correlation of crassicausine with crassicauline A [5]. Because the 8-acetyl group of C_{19}-diterpenoid alkaloids is readily replaceable by an alkoxyl group by treatment with the corresponding alcohol (16), crassicauline A was heated with MeOH at 135-145° for 9 h. The product was shown to be identical in all respects with crassicausine, confirming structure **6** for this alkaloid.

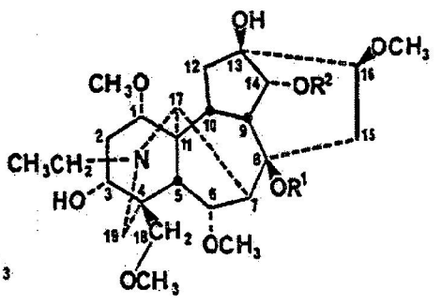
The structure of forestine was confirmed by correlation with crassicauline A [5]. Heating a mixture of crassicauline A perchlorate and H_2O in a sealed glass tube at 140-145° for 8 h afforded a compound identical in all respects with forestine [9]. The ^{13}C-

nmr assignments of C(7) and NCH_2 in forestine are corrected based on its ^{13}C DEPT spectrum.

Crassicautine [**11**] is an amorphous compound having one spot on tlc; $[\alpha]^{24}D$ +26.1° (*c* 0.18, $CHCl_3$); $C_{34}H_{49}NO_{10}$ (based on hrms); ir 3510(OH), 1715(ester), 1609, 1512, 770(aromatic); ^1H nmr δ 1.10(3H, t, J=7 Hz, NCH_2CH_3), 3.00, 3.27, 3.54 (each 3H, s, 3×OCH_3), 3.30 (6H, s, 2×OCH_3), 3.87 (3H, s, aromatic methoxyl), 4.05 (1H, dd, J_1=6 Hz, J_2=1 Hz, 6β-*H*), 4.89 (1H, d, J=4.5 Hz, 14β-*H*), 6.94, 8.07 (4H, A_2B_2 type, OCO-C_6H_4-); ms *m/z* 631(M^+), 616($M-CH_3$), 600($M-OCH_3$, 63.1%), 135(*p*-CO-C_6H_5-OCH_3, 100). ^{13}C-nmr data for crassicautine are given in Table 1.

The ir, ^1H- and ^{13}C-nmr spectra of crassicautine are very similar to those of crassicausine [**6**]; the difference of 16 mass units indicated that crassicautine possesses one more hydroxyl group. Comparison of its ^1H-nmr spectrum with that of crassicausine revealed that the two-proton multiplet at δ 1.5-1.8 due to the methylene at C(3) for crassicautine is not present. Also many features of the ^{13}C-nmr spectrum resemble those of yunaconitine [**10**] and pseudaconine [**12**]. In particular, the signal at 71.8 ppm compared with the value of 34.8 ppm for crassicausine [**6**] indicates a hydroxyl at C(3) in crassicautine.





5 R^1=Ac, R^2=As (crassicauline A)
6 R^1=CH_3, R^2=As (crassicausine)
7 R^1=CH_3, R^2=H
8 R^1=R^2=H (bikhaconine)
9 R^1=H, R^2=As (forestine)

10 R^1=Ac, R^2=As (yunaconitine)
11 R^1=CH_3, R^2=As (crassicautine)
12 R^1=R^2=H (pseudaconine)

In order to prove the above deduction, the correlation of crassicautine A with yunaconitine [**10**] was carried out. Heating **10** with MeOH at 140-145° for 9 h gave an amorphous compound [**11**] that proved to be identical in all respects with crassicautine. Therefore, crassicautine must have structure **11**.

The tlc examination of the crude extract of *A. crassicaule* (not treated with MeOH) shows the existence of crassicausine and crassicautine in the total alkaloid mixture. This result excludes the possibility that crassicausine and crassicautine are artifacts.

The ^{13}C-nmr chemical shifts and assignments for new alkaloids **1**, **6**, and **11** and related compounds **2-5**, **7-10**, and **12** are summarized in Table 1.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Melting points were determined on the Thomas-Kofler block (corrected), ir spectra on a Perkin-Elmer model 1420 spectrophotometer; ^1H-nmr spectra on a Varian EM-390, 90 MHz spectrometer; ^{13}C-nmr spectra on a JEOL model FX-60 spectrometer in $CDCl_3$ with TMS as an internal reference. Mass spectra were measured on a ZAB VG analytical mass spectrometer. A polyvinyl sulfonic acid ion exchange resin (H^+-form, cross linking 1×1.1, from Huabei Pharmaceutical works, China) was used in the separation of total alkaloids from the percolate.

PLANT MATERIAL.—Roots of *A. crassicaule* were collected in Yunlong (Yunnan), China, and the

plant was identified by Professor W.T. Wang, Institute of Botany, Academia Sinica, Beijing, China. A voucher specimen is deposited in that institute.

EXTRACTION AND SEPARATION.—According to the published method (4), 3,200 g of powdered roots of *A. crassicaule* was percolated with 0.05 N HCl until about 3.5 liters was collected. A column of 6,400 g of wet resin (dry weight 640 g) was used to treat the percolate. After exchange, the resin was washed repeatedly on a suction filter with deionized H_2O, spread out, and air-dried overnight. The resin was mixed well with 3,800 ml of 10% NH_4OH, placed in a Soxhlet appartus and then extracted with two portions of Et_2O (500 ml ×2) for 1 h and 3 h, respectively. Evaporation of the Et_2O extracts gave 79.7 g of total alkaloids as a white foam. This mixture (28 g) was chromatographed on a column containing 360 g of Merck alumina 60 PF254 +365 (Type E) and eluted successively with hexane, hexane-2% EtOAc, hexane-8% EtOAc, EtOAc, EtOAc-2% MeOH, MeOH (400-500 ml fractions) to afford fractions A (0.047 g), B (14.360 g), C (2.257 g), D (1.136 g), E (0.571 g), F (2.947 g), G (0.811 g), and H (1.322 g).

ISOLATION OF CHASMANINE [3].—Crystallization of fraction D from hexane containing a few drops of MeOH gave colorless needles (107 mg) showing one spot on tlc (silica gel GF254, $CHCl_3$-10% MeOH), mp 85-87° [lit., (17), mp 90-91°]; $[\alpha]^{29}D$ +35.8° (*c* 0.46, EtOH), which were identified as chasmanine [3] by comparison of the ^{13}C-nmr data with those reported (18).

ISOLATION OF CRASSICAUDINE [1] AND FORESACONITINE [2].—Fraction B (14.36 g) was chromatographed on a column containing 120 g of silica gel 60 and eluted successively with hexane, hexane-10% EtOAc, hexane-20% EtOAc, hexane-40% EtOAc, hexane-60% EtOAc, EtOAc, CH_2Cl_2-10% MeOH, MeOH (400-500 ml fractions) to give fractions B1 (0.551 g, no alkaloidal material), B2 (0.168 g), B3 (0.864 g), B4 (2.582 g), B5 (4.735 g), B6 (0.814 g), B7 (0.820 g), and B8 (0.990 g). Fraction B3 was separated on a Chromatotron (19) (silica gel GF254, 1 mm) and successively eluted with $CHCl_3$ containing increasing amounts of EtOAc (10-100%) and CH_2Cl_2 containing increasing amounts of MeOH (0.2%-10%). Combination of similar fractions gave fractions B3-1 (223 mg), B3-2 (104 mg, crude crassicauline A), B3-3 (29 mg), and B3-4 (588 mg). Fraction B3-4 (274 mg) was separated on seven preparative tlc plates (silica gel GF254, 3 mm, (20×20 cm^2), $CHCl_3$-5% MeOH-25% EtOAc, developing each plate 3 or 4 times to give the fractions B3-4A (290 mg), B3-4B (84 mg), B3-4C (73 mg), and B3-4D (11 mg). Finally, fraction B3-4C was chromatographed on a Chromatotron (19) over alumina GF254 with hexane containing increasing amounts of Et_2O (5-30%) to give crude foresaconitine (33 mg) and crassicaudine [1] (20 mg). The crude foresaconitine was crystallized from Et_2O to give about 20 mg of colorless needles, mp 148-152°; $[\alpha]^{27}D$+26.1° (*c* 0.41, $CHCl_3$), shown to be identical with foresaconitine [2] (5) by comparison of its ^{13}C-nmr spectra, tlc, mmp, and ir spectra with those of an authentic sample. The crude crassicaudine was crystallized from Et_2O to give 15 mg of colorless needles [1], mp 148-150°. Hrms 597.32871. $C_{34}H_{47}NO_8$ requires 597.33017.

HYDROLYSIS OF CRASSICAUDINE [1].—Crassicaudine (33 mg) was dissolved in 5 ml of 10% methanolic NaOH and allowed to stand at room temperature overnight. Removal of solvent under reduced pressure gave a residue which was mixed with a small amount of H_2O and extracted with CH_2Cl_2 (15 ml ×7). The extracts were dried over Na_2SO_4 and evaporated to give a yellowish residue (33 mg) which was purified by preparative tlc over alumina (20 ×20 cm^2, 3 mm, Et_2O) to give a compound (25 mg) showing a single spot on tlc. This compound was identical with chasmanine [3] (18) by comparison of its ^{13}C nmr, ir spectra, and tlc behavior with those of an authentic sample.

PREPARATION OF 14-BENZOYLCHASMANINE [3a].—To a solution of chasmanine (140 mg) in 1 ml of pyridine was added 7 drops of benzoyl chloride. The solution was allowed to stand at room temperature for 18 h. To the residue obtained on evaporation of solvent was added 15 ml of H_2O; the mixture was basified with NH_4OH to pH 11 and then extracted with CH_2Cl_2 (30 ml ×5). The combined extracts were dried over Na_2SO_4. The residue (199 mg) obtained on evaporation of solvent was separated on a Chromatotron (silica gel GF-254, 1 mm) eluting with $CHCl_3$ containing increasing amounts of MeOH (6-10.5%) to give 14-benzoylchasmanine (54 mg) [3a] in 20% yield. ^1H nmr δ 1.20 (3H, t, J=7 Hz, NCH_2CH_3), 3.15, 3.27 (each 3H, s, 2×OCH_3), 3.24 (6H, s, 2×OCH_3), 4.15 (1H, dd, J_1=6 Hz, J_2=1 Hz, 6β-H), 5.09 (1H, t, J=4.5 Hz, 14β-H), 7.25-8.10 (5H, m, aromatic protons). Structure **3a** was assigned on the basis of ^1H- and ^{13}C-nmr spectra.

SYNTHESIS OF CRASSICAUDINE (8-ACETYL-14-BENZOYLCHASMANINE) [1].—A solution of chasmanine (230 mg) in 3 ml of a mixture of dry pyridine and CH_2Cl_2 (1:1) was allowed to stand in a refrigerator for 12 h. To the residue obtained on evaporation of solvent was added 15 ml of H_2O; the mixture was basified with NH_4OH to pH 11 and then extracted with CH_2Cl_2 (30 ml ×5). The combined extracts were dried over Na_2SO_4, evaporated to give a brownish foam (254 mg) containing 14-benzoylchasmanine [5] as the major component in about 90% yield. This residue, dissolved in 1 ml of acetyl chloride plus a little *p*-toluenesulfonic acid, was sealed in a glass tube, and heated on an oil bath at 44° with stirring for 20 h.

Work up gave a pale brown residue which was separated on a Chromatotron of silica gel GF-254 (thick 1 mm) eluting with $CHCl_3$ containing increasing amounts of MeOH (33%) to give a foamy residue (256 mg). Separation of this residue on preparative tlc of alumina (20 ×20 cm^{-2}×3), eluting with a solvent system of hexane-30% Et_2O, gave a crude 8-acetyl-14-benzoylchasmanine (186 mg) in 69.1% yield. Crystallization of the crude 8-acetyl-14-benzoylchasmanine from Et_2O plus a little MeOH gave 102 mg of colorless needles having one spot on tlc. This compound was identical with natural crassicaudine [**1**] by direct comparison of their tlc, mmp, specific rotation, ir, and ^{1}H- and ^{13}C-nmr spectra.

ISOLATION OF CRASSICAULINE A [**5**] AND YUNACONITINE [**10**].—Fraction B5 (4.735 g) was crystallized from Me_2CO/MeOH to give granular crystals (4.069 g) that were recrystallized from the same solvent mixture to afford granular crystals (3.792 g) having one spot on tlc, mp 160-164°; $[\alpha]^{25.5}$D +33.4° (*c* 0.625, $CHCl_3$). This compound was identified as crassicauline A (20) by its ^{13}C-nmr spectral data.

Fraction B7 (0.820 g) was a yellowish, amorphous substance, which was crystallized from Et_2O containing a little Me_2CO to give granular crystals, mp 140-143°; $[\alpha]^{27}$D +30.6° (*c* 0.47, $CHCl_3$). This compound was identified as yunaconitine [**10**] (11) by its ^{13}C-nmr spectrum.

ISOLATION OF CRASSICAUSINE [**6**] AND CRASSICAUTINE [**11**].—Fraction B8 (0.990 g) was chromatographed on preparative tlc (silica gel GF254, 20×20 cm^2×7, 3 mm, $CHCl_3$-10% MeOH, 8-10 times development) to afford the fractions B8-1 (3 mg), B8-2 (52 mg), B8-3 (157 mg), B8-4 (170 mg), B8-5 (205 mg), B8-6 (149 mg). Fraction B8-4 was separated over silica gel GF254 on a chromatotron with hexane-50% $CHCl_3$, hexane-90% $CHCl_3$, and $CHCl_3$ containing increasing amounts of MeOH (2-20%) to give the fractions B8-4A (52 mg), B8-4B (81 mg), and B8-4C (35 mg). Fraction B8-4B was separated again on a Chromatotron over alumina 60 GF254 with Et_2O containing increasing amounts of MeOH (0,0.5, 5%) to give the fractions B8-4B-1 (6 mg), B8-4B-2 (crassicausine, **6**, 24 mg), B8-4B-3 (30 mg), and B8-4B-4 (crassicautine, **11**, 23 mg). Hrpms of crassicausine: 615.34044. $C_{34}H_{49}NO_9$ requires 615.34073. Hrpms of crassicautine: 631.33807. $C_{34}H_{49}NO_{10}$ requires 631.33565.

ISOLATION OF FORESTINE [**9**].—Fractions B8-4A, B8-4B, B8-4B-1, B8-4B-3 with similar fractions obtained from B7-5 were combined and separated by preparative tlc over alumina GF254 (20×20 cm^2×7, 3 mm, Et_2O) to give amorphous forestine (15) (99 mg), having one spot on tlc. It was identified by direct comparison of its ^{13}C nmr, ir spectra, and tlc with those of an authentic sample.

CONVERSION OF CRASSICAULINE A [**5**] TO FORESTINE [**9**].—Crassicauline A (1 g) was dissolved in 10 ml of 70% EtOH with warming, and perchloric acid was added to pH 4 (Congo Red). Evaporation of the solution gave a white residue (1.25 g). The residue (500 mg) was added to 4 ml of distilled H_2O in a sealed glass tube, and the mixture was heated at 140-145° for 8 h. When cooled, colorless needles of crassicauline A perchlorate (11 mg) separated. The mother liquor was basified with NH_4OH to pH 10, extracted with CH_2Cl_2, dried (Na_2SO_4), and evaporated to give a pale yellow residue (260 mg) which was separated by preparative tlc [alumina, (20×20 cm^2)×5, Et_2O-20% hexane] to give crude material corresponding to forestine (121 mg). This material was separated again by preparative tlc as well as on a small column of silica gel (72-325 mesh), eluting with CH_2Cl_2 and MeOH to give an amorphous compound (42 mg), $[\alpha]^{28}$D +55.7° (*c* 0.42, $CHCl_3$) that proved to be identical with forestine [**9**] (15) by comparison of its ^{13}C nmr, ir spectra, and tlc with those of an authentic sample.

CONVERSION OF CRASSICAULINE A [**5**] TO CRASSICAUSINE [**6**].—A solution of crassicauline A (100 mg) in 5 ml of MeOH was sealed in a glass tube and heated in an oil bath at 135-145° for 9 h. Evaporation of the mixture gave a white residue. To this a small amount of H_2O was added; the mixture was basified with NH_4OH to pH 11, extracted with $CHCl_3$ (15 ml×4), dried over Na_2SO_4, and evaporated to dryness to afford a white residue (99 mg). The latter was purified on a Chromatotron [silica gel GF254, 1 mm, eluting with $CHCl_3$ containing increasing amounts of MeOH (1 to 50%)], followed by passing through a column (6 mm diameter) of 1 g silica gel covered with 1 g of alumina to give an amorphous compound (32 mg) having one spot on tlc (alumina, Et_2O-10% hexane), $[\alpha]^{23}$D +32.0° (*c* 0.19, $CHCl_3$) [crassicausine: $[\alpha]^{23}$D +32.7° (*c* 0.20, $CHCl_3$)]; ir 3500 (OH), 1715 (ester), 1609, 770 (cm^{-1}) (aromatic). This compound is identical with crassicausine [**6**] based on ir, ^{1}H- and ^{13}C-nmr spectral data.

PREPARATON OF COMPOUND [**7**].—A solution of crassicauline A (200 mg) in 5 ml of MeOH was sealed in a glass tube, heated in an oil bath at 135-145° for 9 h, and then evaporated to dryness under the reduced pressure to give a white foam. To this 5 ml of 10% NaOH in MeOH was added, and the mixture was allowed to stand at room temperature overnight. After completion of reaction (by monitoring with tlc), the reaction mixture was evaporated; to this a small amount of H_2O was added, the mixture was extracted with $CHCl_3$ (20 ml×5), and evaporated to give a white residue (117 mg). This residue was separated on a Chromatotron (alumina, 1 mm, eluting subsequently with Et_2O, $CHCl_3$, $CHCl_3$-5% MeOH and $CHCl_3$-50% MeOH) to give an amorphous compound [**7**] (44 mg) having one spot on tlc. ^{1}H nmr δ 1.03 (3H, t, J=7 Hz, NCH_2CH_3), 1.44-1.72 (2H, m, 3-H_2), 3.20, 3.21, 3.30, 3.32, 3.45 (each 3H, s, 5× OCH_3), 4.13 (1H, dd, J_1=6 Hz, J_2=1 Hz, 6β-*H*). For ^{13}C-nmr data see Table 1.

PREPARATION OF BIKHACONINE [8].—To 99 mg of crassicauline A [5] was added 4 ml of 10% NaOH in MeOH. This mixture was allowed to stand at room temperature for 3 days and then was evaporated to give a residue (83 mg) that was separated on a Chromatotron [silica gel GF254, 1 mm, eluting with $CHCl_3$ containing increasing amounts of MeOH (10-50%)]. The amorphous product (53 mg) was purified again on a Chromatotron [alumina, 1 mm, eluting with $CHCl_3$ containing increasing amounts of MeOH (10-50%)] to obtain a white amorphous compound (32 mg) that proved to be bikhaconine [8] on the basis of ^{1}H- and ^{13}C-nmr (Table 1) analysis.

CONVERSION OF YUNACONITINE [10] TO CRASSICAUTINE [11].—A mixture of yunaconitine (70 mg) and 5 ml of MeOH was sealed in a glass tube, heated in an oil bath at 140-145° for 9 h, and evaporated to give a residue. To this a little H_2O was added, the mixture was basified with NH_4OH to pH 11, extracted with $CHCl_3$ (15 ml×4), dried over Na_2SO_4, and evaporated to dryness to afford a white residue (67 mg). This residue was separated on a Chromatotron [silica gel GF254, 1 mm, eluting with $CHCl_3$ containing increasing amounts of MeOH (5-50%)] to give an amorphous compound (57 mg) having one spot on tlc, $[\alpha]^{24}D$ +21.3° (*c* 0.15, $CHCl_3$, less than natural crassicautine possibly due to impurities) [crassicautine: $[\alpha]^{24}D$ +26.1° (*c* 0.18, $CHCl_3$)]; ir 3510 (OH), 1715 (ester), 1609, 1512, 770 (aromatic). This compound was identified as crassicautine [11] by direct comparison of ir, ^{1}H- and ^{13}C-nmr spectra with those of natural crassicautine.

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