Immunological Detection and Isolation of a New Taxoid from the Stem Bark of *Taxus baccata*

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The separation of a stem-bark extract of *Taxus baccata* L. cv. stricta by HSCCC resulted in several chromatographic fractions that were analyzed by ELISA using specific *anti*-10-deacetylbaccatin III antibodies. In addition to 10-deacetylbaccatin III and to four known xylosyl taxoids, a new taxoid, 9-deacetyl-9-benzoyl-10-debenzoyltaxchinin A (**1**), was isolated, and its structure was elucidated using spectroscopic methods. The cross-reactivity of **1** with *anti*-10-deacetylbaccatin III antibodies was examined.

We previously reported on the combination of chromatographic methods and enzyme-linked immunosorbent assay (ELISA) in the search for new taxoids whose structures are closely related to Taxol (paclitaxel).[1] In this respect, the chromatographic fractions were monitored by ELISA using *anti*-Taxol antibodies.[2] The same strategy using *anti*-10-deacetylbaccatin III antibodies[3] was applied in the search for new taxoids lacking a side chain at C-13. The cross-reactivity study showed that the latter antibodies had a high affinity to 10-deacetylbaccatin III and its very closely related compounds, such as baccatin III. In contrast, taxoids bearing a side chain at C-13, such as Taxol and cephalomannine, are less recognized by this antiserum (0.2% cross-reactivity).[3] The taxoids lacking the side chain substituent are of interest because the examination of their structures could contribute to a better understanding of the complex taxoid biosynthetic pathways. In some cases, such compounds could serve as precursors for the hemisynthesis of bioactive taxoids, such as Taxol and Taxotere (docetaxel).[4−6] In this report, we describe the immunodetection and isolation of a new taxoid from *T. baccata* stem-bark extract.

After solvent partition of the crude MeOH extract, the CH_2Cl_2 part was submitted to Si gel column chromatography, and the fractionation was monitored by ELISA. The fraction eluted with $CHCl_3$-MeOH (95:5), which had high affinity to *anti*-10-deacetylbaccatin III antibodies, was further separated by high-speed countercurrent chromatography (HSCCC). The 24 fractions obtained from HSCCC were analyzed by ELISA. As shown in Figure 1, fraction 2 exhibited the highest immunosignal. The occurrence of 10-deacetylbaccatin III in this fraction was confirmed by an authentic standard spike on HPLC. On the other hand, fraction 3, which showed a relatively high immunosignal, was purified by preparative TLC to give a new taxoid (**1**).

The FABMS of compound **1** exhibited the ions at *m/z* 665 and 573, corresponding to $[M + \text{glycerine}]^+$ and $[M + H]^+$, respectively. So the molecular formula was determined to be $C_{31}H_{40}O_{10}$. The fragments at *m/z* 555 and 537 corresponded to the loss of one and two molecules of H_2O from the molecular weight, respectively. The NMR data of compound **1** obtained in CD_3OD are shown in Table 1. The ^{1}H-NMR signals at δ 5.03 and 5.44 (1H each, br s) and ^{13}C-NMR signals at δ 149.0 (quaternary carbon) and 114.4 ($=CH_2$) suggested the presence of an exomethylene moiety. The absence of long-range correlation in the HMBC spectrum between Me-16 (or Me-17) with C-11 and the ^{13}C-NMR signal of C-15 at δ 76.7 showed that **1** was a taxoid of the abeo-11(15→1)-type.[7] The presence of a benzoate was confirmed by the ^{1}H-NMR signals at δ 7.92 (2H), 7.58 (1H), and 7.44 (2H). Both ^{1}H- and ^{13}C-NMR spectra revealed two acetyl groups. The presence of acetates at C-2 and C-7 and a benzoate at C-9 was proved by analysis of the ^{1}H- and ^{13}C-NMR spectra and the HMBC spectrum. The HMBC experiments related the benzoate carbonyl group both to the *ortho* protons at δ 7.92 and to the H-9 at δ 5.18; the experiments also related one of the acetate carbonyl groups to the acetate methyl group at δ 1.89 and to the H-7 at δ 4.81 and the other to the acetate methyl at δ 1.98 and to the H-2 at δ 5.91. The assignment of the signal at δ 5.18 to H-9 rather than to H-10 was made on the basis of HMBC correlation of the H-9 with C-3, C-7 and the signal of

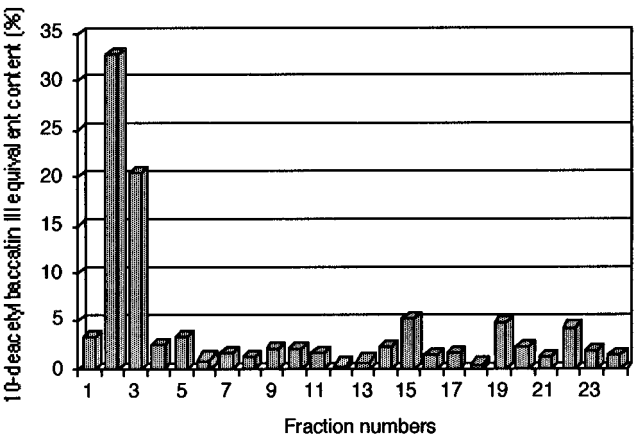

Figure 1. Distribution of immunoreactive constituents in fractions from HSCCC.

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Table 1. The NMR Data of Compound **1** (δ, CD_3OD)

position	^{1}H-NMR δ, J in Hz	^{1}H−^{1}H COSY	^{13}C-NMR δ	HMBC
1			67.8	
2	5.91, 1H, d, J = 9.1	H-3	72.0	C-1, C-3, C-8, C-14, C-15, *C*OMe
3	3.29, 1H, d, J = 9.1	H-2	46.6	C-1, C-2, C-4, C-5, C-8, C-9, C-20, Me-19
4			149.0	
5	4.64, 1H, brt, J = 7.6	H-6α, β	66.9	
6	α 1.81, 1H, m, $\sum J$ = 23.0	H-5, H-6β, H-7	36.9	C-5, C-7, C-8
	β 2.02, 1H, m, $\sum J$ = 31.0	H-5, H-6α, H-7		C-4
7	4.81, 1H, t, J = 7.7	H-6α, β	73.5	C-8, Me-19, *C*OMe
8			45.4	
9	5.18, 1H, d, J = 3.8	H-10	77.8	C-3, C-7, C-8, C-10, C-11, *C*OPh
10	4.80, 1H, d, J = 3.8	H-9	69.3	C-1, C-8, C-9, C-11, C-12
11			135.6	
12			150.0	
13	4.51, 1H, t, J = 7.1	H-14α, β	78.1	C-11, C-12
14	α 2.11, 1H, m, J = 7.6, 14.5	H-13, H-14β	41.7	C-1, C-2, C-13, C-15
	β 2.30, 1H, m, J = 7.1, 14.5	H-13, H-14α		C-1, C-11, C-12, C-15
15			76.7	
Me-16	1.23, 3H, s	Me-17	28.4	C-1, C-15, Me-17
Me-17	1.45, 3H, s	Me-16	27.1	C-1, C-15, Me-16
Me-18	1.66, 3H, s		13.2	C-11, C-12, C-13
Me-19	1.06, 3H, s		15.3	C-3, C-7, C-8, C-9
20	a 5.03, 1H, brs	H-20b	114.4	C-3, C-5
	b 5.44, 1H, brs	H-20a		C-3, C-5
7-CO*Me*	1.89, 3H, s		21.2	*C*OMe
2-CO*Me*	1.98, 3H, s		22.0	*C*OMe
7-*C*OMe			172.1	
2-*C*OMe			172.5	
*C*OPh			167.1	
1′			131.3	
2′, 6′	7.92, 2H, d, J = 7.1	H-3′, H-4′, H-5′	131.0	*C*OPh, C-1′, C-4′
3′, 5′	7.44, 2H, t, J = 7.5	H-2′, H-4′, H-6′	129.9	C-1′, C-2′, C-4′, C-6′
4′	7.58, 1H, td, J = 7,4	H-2′, H-3′, H-5′, H-6′	134.7	C-2′, C-3′, C-5′, C-6′

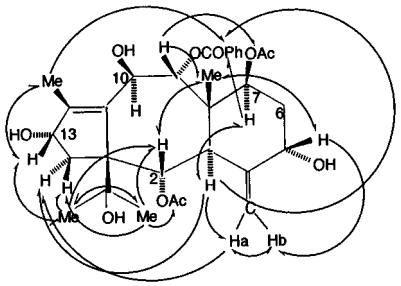


Figure 2. Key NOEs observed for compound **1**.

H-10 at δ 4.80 with C-1, C-12. The same C-9 and C-10 substituent pattern has been also observed in a brevifoliol derivative (**2**).[8]

A twist-chair conformation was assigned to the ring B in compound **1**; the oxygen functions at C-9 and C-10 are pseudoaxial and at H-9 and H-10 are pseudoequatorial ($J_{9,10}$ = 3.8 Hz). This arrangement is different from that found in taxoids where ring B has a twist-boat conformation and H-9 and H-10 are pseudoaxial ($J_{9,10} \approx$ 10 Hz)[7] but is similar to the chairlike conformation of taxchinin D.[9]

Most ^{1}H-NMR data were comparable to those of taxchinin A (**3**),[10] except the arrangement at C-9 (H-9 δ from 6.02 to 5.18) and C-10 (H-10 δ from 6.56 to 4.80). Another difference was the upfield shift of Me-18 (δ from 2.11 to 1.66), which might be due to its stereo position below the plane of the benzene ring. The stereochemistry of compound **1** was further determined by NOE difference spectroscopy. The results are summarized in Figure 2. Thus, the structure of compound **1** was assigned as 9α-benzoxy-2α,7β-diacetoxy-5α,10β,13α,15-tetrahydroxy-11(15→1)-abeotaxa-4(20),11-diene, or 9-deacetyl-9-benzoyl-10-debenzoyltaxchinin A.

In comparison with spectra recorded in CD_3OD, compound **1** showed very broad unresolved ^{1}H-NMR signals in $CDCl_3$ at room temperature. The same observation was reported by Barboni *et al.*[11] and could probably be due to a slow equilibrium between two or more conformational isomers. In our case, we observed two conformers with a ratio of 5:3. At −5 °C (the lowest temperature of our valuable temperatures studied), we were unable to measure the coupling constant for the two conformers. This precluded a complete conformational analysis. At +61 °C, most of the signals were sharpened, whereas the others remained broad. A detailed study at suitable temperatures is in progress.

Interestingly, fractions 4−13 of HSCCC could be crystallized in the HSCCC solvent system. Fractions 5, 9, and 12 contained almost pure compounds (90% purity on HPLC); these compounds were identified by their spectroscopic data as 7β-xylosyl-10-deacetyltaxol C (132 mg, yield of 0.015%), 7β-xylosyl-10-deacetyltaxol (85 mg, yield of 0.010%), and 7β-xylosyl-10-deacetylcephalomannine (63 mg, yield of 0.007%). In addition, 7β-xylosyl-10-deacetyltaxol D (3 mg, yield of 0.0003%) was identified in fractions 14−19. We have previously reported the isolation of these four xylosyl taxoids using HPLC.[12] It was laborious to purify them by HPLC. However, relatively pure xylosyl taxoids could be obtained after only one course of HSCCC, and their yields were higher than those from HPLC. This fact demonstrated that HSCCC is a useful tool for taxoid purification.

Studies on cross-reactivity of the isolated pure compounds (**1** and the four xylosyl taxoids) with *anti*-10-deacetylbaccatin III antibodies are graphed in Figure 3. In comparison to 10-deacetylbaccatin III, the antibodies employed had a lower affinity to compound **1**.

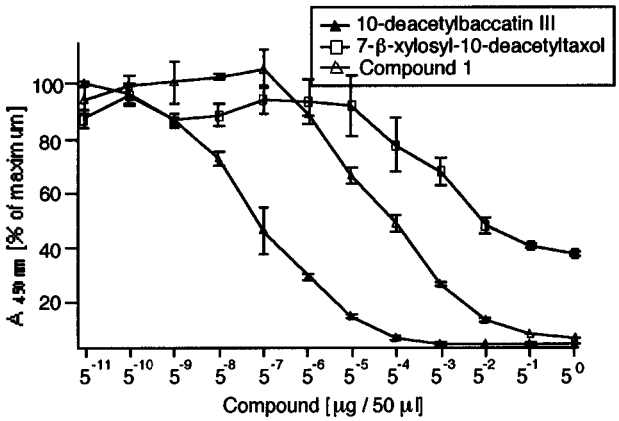
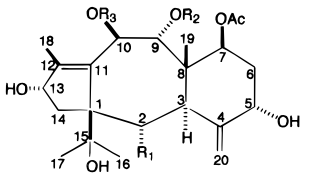


Figure 3. Cross-reactivity of compound **1**, 7β-xylosyl-10-deacetyltaxol, and 10-deacetylbaccatin III with antiserum.

This circumstance could be explained by the significant structural differences between them. The four xylosyl taxoids were less recognized by the antibodies employed than was compound **1**. These xylosyl taxoids have similar affinity to the antibodies employed as do Taxol and cephalomannine.[3] The curve of 7β-xylosyl-10-deacetyltaxol shown in Figure 3 represents the same profile as that of other xylosyl taxoids.



	R_1	R_2	R_3
1	OAc	COPh	H
2	H	COPh	H
3	OAc	OAc	COPh

The results of our previous investigation[1] and this report clearly demonstrate that the use of specific antibodies for the monitoring of chromatographic fractions is an efficient method for a rapid detection and isolation of target taxoids.

Experimental Section

General Experimental Procedures. Optical rotation was determined on a Perkin-Elmer 141 polarimeter. The UV spectrum was obtained on a Shimadzu UV-265 FS spectrophotometer. The IR spectrum was recorded as a KBr pellet on a Shimadzu IR-470 spectrophotometer. ^{1}H- and ^{13}C-NMR spectra were recorded on a Varian-Unit 600. Chemical shift data are given in ppm downfield from TMS as internal reference. The DEPT sequence was used to distinguish the methylene carbon signals from those due to methine, Me, and quaternary carbons; one-bond ^{1}H−^{13}C and ^{1}H−^{1}H connectivities were determined via 2D proton-detected HMQC and COSY experiments, respectively; two- and three-bond ^{1}H−^{13}C correlations were determined using 2D proton-detected HMBC experiments optimized for $J_{CH} = 5$ and 10 Hz. The FABMS were obtained on a Kratos MS9 spectrometer using glycerol as matrix. Si gel 60 and Lichroprep RP-18 (Merck, mean particle size, 0.040−0.063 mm) were used for column chromatography.

Plant Material. Stem-bark samples of *T. baccata* L. cv. stricta (Taxaceae) were collected from trees growing in the Jardin Expérimental Jean Massart of the Université Libre de Bruxelles, Belgium. A voucher specimen is located in the Laboratory of Plant Morphology of U.L.B.

Extraction and Purification Procedure. The dried stem barks (880 g) were macerated for 48 h with MeOH. The syrup remaining after evaporation (*ca.* 140 g) was extracted first with petroleum (4 × 200 mL) and then with CH_2Cl_2 (4 × 200 mL). The CH_2Cl_2 residue (ca. 10 g) was adsorbed on cellulose 2100 ff (Macherey-Nagel, Düren, Germany) (10 g), submitted to Si gel column chromatography (100 g), and eluted using a stepwise gradient of $CHCl_3$ in petroleum (in increments of 10%) until pure $CHCl_3$ was reached and then with increased concentrations of MeOH in $CHCl_3$ until pure MeOH was achieved. The fraction (360 mg) eluted with $CHCl_3$−MeOH (95:5), showing high affinity to *anti*-10-deacetylbaccatin III antibodies, was further purified by HSCCC.

High Speed Counter Current Chromatography. The HSCCC was performed using an Ito multilayer coil separator extractor (P.C., Inc., Potomac, MD) equipped with a 66 m × 2.6 mm i.d. column (column capacity: 350 mL). The solvent system was modified from our previous report,[13] petroleum-EtOAc-MeOH-H_2O (4:12:4:5, v/v). The pertinent fraction (360 mg) was dissolved in 10 mL of a 1:1 (v/v) mixture of the two phases of the solvent system, and the solution was filtered before it was loaded into the column. The separation was performed at room temperature and monitored by on-line TLC;[14] 24 fractions were obtained. The purification of compound **1** from fraction 3 was achieved by preparative TLC using $CHCl_3$−MeOH (9:1).

High Performance Liquid Chromatography. The HPLC separation was achieved on Hibar pre-packed column Lichrosorb RP-18 (mean particle size 7 μm, 250 mm × 4 mm i.d.), equipped with a guard column (LiChrospher 100, RP-18, 5 μm), using MeCN−H_2O−MeOH (30:60:10) as mobile phase at a flow rate of 1 mL/min. The apparatus (Hewlett-Packard 1040A HPLC detection system, with a Waters pump Model 510 and a Waters Injector Model U6K) was equipped with a photodiode-array detector. The t_R of 10-deacetylbaccatin III was 5.6 min.

ELISA Procedure. The ELISA method used in this study was described previously.[3] The *anti*-10-deacetylbaccatin III antibodies were obtained by immunizing rabbits against 10-succinyl-10-deacetylbaccatin III−bovine serum albumin conjugate. Aliquots from each chromatographic fraction assayed by ELISA were first dissolved in MeOH, and a suitable volume of this solution was then diluted in an appropriate buffer for ELISA.

9-Deacetyl-9-benzoyl-10-debenzoyltaxchinin A (1): white powder, 20 mg (yield of 2.3 × 10^{-3}%); $[\alpha]^{20}_D$ +19.4° (*c* 1.26, MeOH); UV (MeOH) λ max (log ϵ) 210 (2.13), 228 (2.20), 274 (1.23), 281 (1.14) nm; IR ν max (KBr) 3385, 2960, 1711, 1433, 1364, 1260, 1171, 1107, 1046, 1021, 709 cm^{-1}; FABMS [M + glycerine]$^+$ 665, [M + H]$^+$ 573, [M − H_2O]$^+$ 555, [M − 2 × H_2O]$^+$ 537; ^{1}H- and ^{13}C-NMR, see Table 1.

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