Identification of Six Taxine Alkaloids from *Taxus baccata* Needles

Louis H. D. Jenniskens, Erik L. M. van Rozendaal, and Teris A. van Beek*

Department of Organic Chemistry, Phytochemical Section, Agricultural University, Dreijenplein 8, 6703 HB Wageningen, The Netherlands

Peter H. G. Wiegerinck and Hans W. Scheeren

Department of Organic Chemistry, NSR Center for Molecular Structure, Design and Synthesis, Toernooiveld, 6525 ED Nijmegen, The Netherlands

Received July 18, 1995[⊗]

Six taxine alkaloids were isolated from *Taxus baccata* needles and partially separated. An HPLC method for the analysis of the taxine fraction was developed. By NMR and deamination/hydrolysis studies it was established that the taxine fraction consisted mostly of taxicin-I and taxicin-II derivatives in a variable ratio. Four of the six taxine alkaloids isolated have not been reported before. All of the alkaloids were found to be unstable under neutral and basic conditions, and some of them were very easily interconverted.

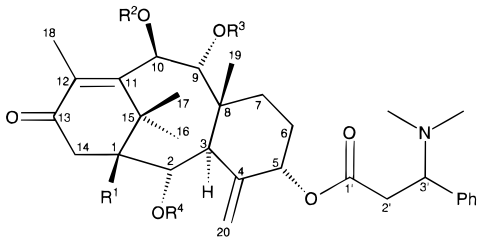
The discovery of the diterpenoid natural product taxol,[1] together with its clinical activity against ovarian, breast, lung, and other cancers,[2] and its unique mechanism of action[3] have created a real boost in phytochemical studies of different *Taxus* species. The first phytochemical study was carried out by Lucas in 1856.[4] He isolated an ill-defined alkaloidal mixture from *T. baccata* L. (Taxaceae), which he called "taxine". Crude taxine is readily isolated by acid extraction, in 0.5−1% yield. The constituents degrade easily: elimination of the dimethylamino function leads to the corresponding cinnamates. Additionally, migration of acetyl groups and photodegradation occur readily.[5]

Although taxine has been described as a mixture of at least 11 compounds, only seven of them have been structurally characterized, probably due to their instability and difficulty of separation. The major alkaloid of the taxine fraction from needles is taxine B (∼20%) (**1**).[6−9] Isotaxine B (**3**) also occurs in appreciable concentrations, while the other five characterized taxines are only minor constituents, occurring in concentrations of about 2%.[10−15] Of these, taxines A[10,11] and C[14,15] have a rearranged carbon skeleton.[12,13]

Taxine B (**1**) and some related alkaloids occur in relatively high concentrations, and this makes them an interesting starting material for semisynthetic studies toward 7-deoxytaxol and related taxoids.[16−18] However, it is difficult to obtain pure taxine B. After an additional acid/base extraction the purified taxine fraction still consists of several constituents according to NMR. This prompted us to a closer investigation, and we report here on the identification of the major taxine alkaloids of *T. baccata*, the problems that arose on handling this material, and the synthetic conversion to the corresponding cinnamates. As no method for the analysis of the taxine mixture has been described so far, the first aim was to develop a simple HPLC method for the analysis.

Results and Discussion

It was found that a 15-cm C18 column in combination with a ternary gradient of 0.05 M sodium dihydrogenphosphate buffer (pH 3.0)−MeCN−MeOH (see Experimental Section) at a flow rate of 2 mL/min gave a satisfactory separation of the taxine alkaloids. The system could not, however, achieve a complete base line separation of all the taxines present in the crude alkaloid mix. The use of a buffer proved to be absolutely essential. In Figure 1, a typical HPLC chromatogram of the taxine fraction of *T. baccata* with UV detection at 280 nm is given. The peaks 1−6 in this chromatogram correspond with the taxines **1**-**6**. The gradient is quite steep at the end of the chromatogram for a quicker elution around 25−26 min of the much less polar cinnamates **10**, which are always present in older or improperly stored taxine samples.



1: R^1 = OH, R^2 = Ac, R^3 = R^4 = H
2: R^1 = H, R^2 = Ac, R^3 = R^4 = H
3: R^1 = OH, R^3 = Ac, R^2 = R^4 = H
4: R^1 = OH, R^3 = H, R^2 = R^4 = Ac
5: R^1 = H, R^3 = Ac, R^2 = R^4 = H
6: R^1 = OH, R^2 = H, R^3 = R^4 = Ac
7: R^1 = OH, R^2 = R^3 = R^4 = H
8: R^1 = OH, R^2 = R^3 = R^4 = Ac
9: R^1 = H, R^2 = R^3 = R^4 = Ac

The cinnamates are formed by elimination of dimethylamine from the side chain of the corresponding taxines. This elimination reaction was observed upon prolonged drying of the needles at 60 °C and when the taxines were dissolved in neutral, basic, or weakly acidic solutions (e.g., HPLC samples in MeOH). Under acidic conditions (acid extraction with 0.5% H_2SO_4 or storage of taxine solutions in low pH buffer/MeOH or buffer/

* To whom correspondence should be addressed. Phone: + 31.317.482376. Fax: + 31.317.484914. E-mail: teris.vanbeek@sg1.oc.wau.nl.

⊗ Abstract published in *Advance ACS Abstracts,* January 1, 1996.

0163-3864/96/3259-0117$12.00/0

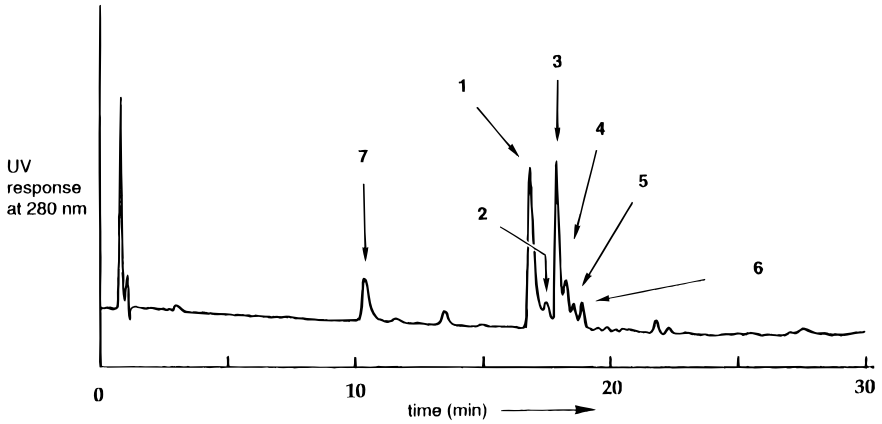


Figure 1. HPLC chromatogram of a freshly prepared crude taxine fraction from *Taxus baccata*. RP-C18 column (15 cm) with buffer-MeCN-MeOH gradient (see Experimental Section). Detection UV at 280 nm. For peak assignment see text.

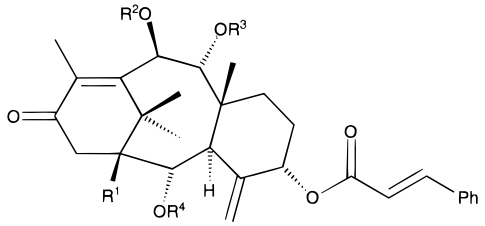
MeCN mixtures), however, no formation of the cinnamates was observed. This latter observation contrasts with what has been suggested or reported.[5,19] In their solid form taxines are stable.

The formation of the cinnamates **10** is also synthetically possible by quaternization of the amino group with MeI followed by treatment with K_2CO_3 in a 1:1 mixture of EtOH and H_2O.[16,18,20] A 4.5-h treatment with 6.5 equiv of K_2CO_3 resulted in the complete hydrolysis of all the acetate groups originally present in **10**, yielding two products with retention times around 23 min. These mild conditions for acetate hydrolysis are superior for the use of sodium methoxide.[16] As with the latter method, partial hydrolysis of the cinnamoyl side chain was also observed.

On the basis of observed instability in MeOH, it was thought that the MeI/K_2CO_3 reactions could perhaps be avoided by simply refluxing the taxine fraction in MeOH. Unfortunately, after a sample of taxines was refluxed in MeOH for 11 h, a mixture was obtained consisting of 10% taxines, 65% cinnamates **10**, and 25% compounds corresponding with two peaks around 23 min (ratio 3:1). The next goal was the full characterization of the latter two compounds that were suspected to be the parent deacetylated and deaminated taxine skeletons. Knowing the identity of these parent skeletons could be of great help in the later structure elucidation of the unstable taxines.

A relatively pure mixture of the two compounds of interest was obtained by treating the crude taxine fraction with MeI/K_2CO_3 followed by a partition step. Separation of the two compounds was carried out by preparative TLC on alumina. The major product was identified as *O*-cinnamoyltaxicin-I (**11**) (for NMR data, see Tables 1 and 2). This is formed from taxine B (**1**) and its isomers by elimination of dimethylamine from the side chain and hydrolysis of the different acetates at C2, C9, and/or C10. Acetylation to the known *O*-cinnamoyltaxicin-I triacetate (**13**) confirmed this identification.[21]

The mass spectrum of the minor product **12** revealed a parent peak [M^+] at m/z 480, corresponding to the composition $C_{29}H_{36}O_6$. As **11** possessed a molecular formula of $C_{29}H_{36}O_7$, this suggested the absence of one hydroxyl group. The major difference between the ^{13}C-NMR spectra of the two compounds was the absence of a singlet for C1 in the alcohol region between 65 and 80 ppm. Instead, a doublet at 51.1 ppm was observed. Furthermore, it was noticed that the signals for C2, C14, C15, C16, and C17 had shifted by several ppm, while all other signals remained relatively unchanged in comparison to **11**. From these observations it was concluded that the minor compound was *O*-cinnamoyltaxicin-II (**12**), the 1-deoxy isomer of compound **11**. This assignment was confirmed by its acetylation to the known *O*-cinnamoyltaxicin-II triacetate (**14**).[21]



10: R^1 = H or OH, R^2 = R^3 = R^4 = H or Ac
11: R^1 = OH, R^2 = R^3 = R^4 = H
12: R^1 = H, R^2 = R^3 = R^4 = H
13: R^1 = OH, R^2 = R^3 = R^4 = Ac
14: R^1 = H, R^2 = R^3 = R^4 = Ac
15: R^1 = OH, R^2 - R^3 = acetonide, R^4 = H
16: R^1 = H, R^2 - R^3 = acetonide, R^4 = H

These results show that the purified taxine fraction is largely made up of different isomers of taxine B (**1**) and 1-deoxytaxine B (**2**). The relatively high natural concentrations, the ease of isolation, and the presence of the hydroxyl function at C1 are advantages of the use of taxine B (and its isomers) as a starting material in a semisynthetic route toward 7-deoxytaxol. The 1-deoxytaxine B isomer(s), although present in somewhat smaller amounts in the taxine fraction, might offer an interesting entrance toward 1,7-dideoxytaxol derivatives. This is especially interesting for structure–activity relationship studies as the hydroxyl group at C1 in taxol is the only substituent whose importance has not yet been clarified.[22] A prerequisite for the use of taxine B and its deoxy derivative is, of course, an efficient separation at some stage.

During the investigations it was found that the ratio of **11** and **12** in the deacetylated deaminated taxine fraction varied between 3:1 and 9:1 for needles of different trees. We believe that this variation is dependent on the *T. baccata* variety. Recently ElSohly *et al.* reported considerable variations in taxol and related taxanes in different *Taxus* species and cultivars.[23]

Table 1. ^{1}H-NMR Data of **1-8**, **11-16** (200 MHz, $CDCl_3$)

atom	Compound **1**[a,b]	**2**	**3**[a,c]	**4**	**5**	**6**	**7**[a]
1		2.38 (dd, 2.4, 6.6)			2.37 (dd, 2.6, 6.8)		
2	3.95 (d, 6.9)	4.13 (dd, 2.4, 6.6)	4.05 (d, 6.9)	5.52 (d, 6.0)	4.22 (dd, 2.6, 6.4)	5.55 (d, 6.0)	3.99 (d, 7.0)
3	3.13 (d, 6.9)	3.02 (d, 6.6)	3.13 (d, 6.9)	3.30 (d, 6.0)	3.03 (d, 6.4)	3.30 (d, 6.0)	3.13 (d, 7.0)
5	5.12 (br s)	5.19 (m)	5.12 (br s)	5.10 (br s)	5.14 (br s)	5.10 (br s)	5.09 (br s)
6	1.56 (m)	n.o.[d]	1.56 (m)	n.o.	n.o.	n.o.	1.64 (m)
7	1.37 (m), 1.73 (m)	n.o.	1.37 (m), 1.73 (m)	n.o.	n.o.	n.o.	1.32 (m), 1.73 (m)
9	4.28 (d, 9.5)	4.20 (d, 9.7)	5.74 (d, 9.5)	4.33 (d, 9.7)	5.66 (d, 9.9)	5.27 (d, 10.0)	4.15 (d, 9.4)
10	5.85 (d, 9.5)	5.80 (d, 9.7)	5.01 (d, 9.5)	5.85 (d, 9.7)	4.97 (d, 9.9)	5.03 (d, 10.0)	4.88 (d, 9.4)
14	2.66 (s)	2.63 (m), 2.77 (m)	2.68 (s)	2.73 (s)	2.50 (m), 2.61 (m)	2.56 (s)	2.59 (d, 19.5), 2.69 (d, 19.5)
16-CH_3	1.52 (s)	1.56 (s)	1.69 (s)	1.54 (s)	1.73 (s)	1.73 (s)	1.59 (s)
17-CH_3	1.25 (s)	1.16 (s)	1.35 (s)	1.20 (s)	1.24 (s)	1.24 (s)	1.34 (s)
18-CH_3	2.20 (s)	2.22 (s)	2.20 (s)	2.16 (s)	2.15 (s)	2.12 (s)	2.08 (s)
19-CH_3	1.13 (s)	1.11 (s)	0.93 (s)	1.07 (s)	0.90 (s)	0.88 (s)	1.11 (s)
20	5.37 (s), 5.41 (s)	5.34 (s)	5.39 (s), 5.42 (s)	4.75 (s), 5.25 (s)	5.35 (s), 5.38 (s)	4.68 (s), 5.26 (s)	5.34 (s), 5.39 (s)
2′	2.53 (dd, 7.0, 16.0), 2.91 (dd, 7.0, 16.0)	2.43 (m), 2.85 (m)	2.52 (dd, 7.0, 16.0), 2.90 (dd,7.0, 16.0)	2.55 (dd, 7.8, 16.0), 2.95 (dd,7.8, 16.0)	2.41 (m), 2.90 (m)	2.55 (dd, 7.8, 16.0), 2.96 (dd, 7.8, 16.0)	2.51 (dd, 6.9, 16.0), 2.88 (dd,6.9, 16.0)
3′	3.86 (t, 7.0)	4.06 (t, 7.5)	3.84 (t, 7.0)	3.76 (t, 7.8)	3.94 (t, 7.5)	3.76 (t, 7.8)	3.81 (t, 6.9)
aromatic	7.29 (m)	7.37 (m)	7.32 (m)	7.30 (m)	7.35 (m)	7.30 (m)	7.29 (m)
1-OH	n.o.		n.o.	n.o.		n.o.	n.o.
2-OH	n.o.	n.o.	n.o.		n.o.		n.o.
9-OH	n.o.	n.o.		n.o.			n.o.
10-OH			n.o.		n.o.	n.o.	n.o.
$N(CH_3)_2$	2.22 (s)	2.29 (s)	2.22 (s)	2.25 (s)	2.25 (s)	2.23 (s)	2.21 (s)
2-OAc				2.11 (s)		2.10 (s)	
9-OAc			2.09 (s)	-	2.09 (s)	2.10 (s)	
10-OAc	2.16 (s)	2.15 (s)		2.11 (s)			
$C(CH_3)_2$							

atom	Compound **8**[a,b]	**11**[a,d]	**12**	**13**[e]	**14**[e]	**15**	**16**
1			2.33 (dd, 2.0, 6.9)		2.23 (dd, 2.1, 6.9)		2.41 (dd, 2.2, 7.0)
2	5.58 (d, 6.8)	4.05 (dd, 6.4, 6.9)	4.19 (m)	5.61 (d, 6.9)	5.55 (dd, 6.3, 2.1)	4.08 (m)	4.24 (m)
3	3.32 (d, 6.8)	3.37 (d, 6.9)	3.19 (d, 6.3)	3.53 (d, 6.9)	3.40 (d, 6.3)	3.19 (d, 6.6)	3.07 (d, 6.3)
5	5.11 (br s)	5.32 (br s)	5.30 (br s)	5.33 (br s)	5.34 (br s)	5.30 (br s)	5.34 (br s)
6	1.53 (m)	1.40 (m), 1.70 (m)	1.74 (m), 2.01 (m)	n.o.	n.o.	1.67 (m)	1.71 (m)
7	1.66 (m)	1.75 (m), 1.82 (m)	1.39 (m), 1.79 (m)	n.o.	n.o.	1.67 (m), 1.95 (m)	1.71 (m), 1.96 (m)
9	5.90 (d, 10.4)	4.17 (dd, 3.8, 9.3)	4.06 (d, 9.1)	5.92 (d, 10.3)	5.88 (d, 10.2)	4.26 (d, 9.3)	4.21 (d, 9.2)
10	6.08 (d, 10.4)	4.92 (dd, 2.4, 9.3)	4.85 (d, 9.1)	6.10 (d, 10.3)	6.04 (d, 10.2)	4.90 (d, 9.3)	4.88 (d, 9.2)
14	2.65 (d, 19.7), 2.79 (d, 19.7)	2.66 (d, 16.0), 2.70 (d, 16.0)	2.23 (d, 19.8), 2.82 (dd, 6.9, 19.8)	2.66 (d, 19.7), 2.90 (d, 19.7)	2.42 (d, 20.0), 2.83 (dd, 6.9, 20.0)	2.67 (br s)	2.21 (d, 19.9), 2.86 (dd, 7.0, 19.9)
16-CH_3	1.69 (s)	1.61 (s)	1.62 (s)	1.80 (s)	1.76 (s)	1.61 (s)	1.70 (s)
17-CH_3	1.22 (s)	1.36 (s)	1.24 (s)	1.23 (s)	1.24 (s)	1.35 (s)	1.24 (s)
18-CH_3	2.26 (s)	2.10 (s)	2.09 (s)	2.27 (s)	2.27 (s)	2.10 (s)	2.12 (s)
19-CH_3	0.89 (s)	1.17 (s)	1.12 (s)	0.94 (s)	0.92 (s)	1.07 (s)	1.07 (s)
20	4.69 (s), 5.29 (s)	5.38 (s), 5.46 (s)	5.37 (s), 5.41 (s)	4.68 (s), 5.35 (s)	4.84 (s), 5.35 (s)	5.37 (s), 5.43 (s)	5.36 (s), 5.42 (s)
2′	2.54 (dd, 7.6, 15.3), 2.91 (dd, 7.6, 15.3)	6.39 (d, 15.9)	6.39 (d, 15.9)	6.42 (d, 15.9)	6.42 (d, 15.9)	6.33 (d, 15.8)	6.36 (d, 16.0)
3′	3.79 (t, 7.6)	7.64 (d, 15.9)	7.62 (d, 15.9)	7.65 (d, 15.9)	7.66 (d, 15.9)	7.61 (d, 15.8)	7.63 (d, 16.0)
aromatic	7.28 (m)	7.43 (m), 7.75 (m)	7.41 (m), 7.73 (m)	7.46 (m), 7.75 (m)	7.43 (m), 7.76 (m)	7.41 (m), 7.71 (m)	7.42 (m), 7.73 (m)
1-OH	n.o.	3.54 (s)		n.o.		n.o.	-
2-OH		2.41 (d, 6.4)	n.o.			n.o.	n.o.
9-OH		2.64 (d, 3.8)	n.o.			n.o.	n.o.
10-OH		2.58 (d, 2.4)	n.o.			n.o.	n.o.
$N(CH_3)_2$	2.23 (s)						
2-OAc	2.08 (s)			2.08 (s)	2.06 (s)		
9-OAc	2.07 (s)			2.06 (s)	2.05 (s)		
10-OAc	2.13 (s)			2.12 (s)	2.07 (s)		
$C(CH_3)_2$						1.42 (s), 1.48 (s)	1.42 (s), 1.48 (s)

[a] 400 MHz. [b] In full agreement with Ettouati et al.[8] [c] In full agreement with van Rozendaal.[17] [d] n.o. = not observable. [e] In full agreement with Ettouati *et al.*[16] [f] In full agreement with Appendino *et al.*[21]

Table 2. ^{13}C-NMR Data of **1**-**5**, **7**, **11**, **12**, **15**, and **16** (50.0 MHz, $CDCl_3$)

		Compound									
atom	mult[a]	**1**[b]	**2**	**3**	**4**	**5**	**7**	**11**[c]	**12**	**15**	**16**
1	s	77.6	51.1(d)	77.9	77.2	51.4(d)	77.8	77.9	51.1(d)	79.0	51.8(d)
2	d	71.6	68.1	71.5**	72.0	68.1	71.5	71.3	67.9	71.7	68.1
3	d	46.7	45.0	46.5	45.7	44.8	46.6	46.5	45.0	45.3	44.0
4	s	143.5	143.8	143.4	142.1	143.3	144.0	143.9	143.9	143.1	143.3
5	d	77.8	78.1*	78.5*	78.2*	78.8*	78.0*	78.1	78.2*	78.3*	78.3*
6	t	28.9	28.8	28.7	28.4	28.6	28.9	28.8	28.8	28.9	28.9
7	t	26.3	26.3	27.5	26.0	27.6	26.3	26.2	26.3	26.8	26.8
8	s	45.4	45.2	44.9	45.1	44.5	45.0	44.8	44.6	42.8	41.1
9	d	75.1	76.8*	77.2*	76.4*	77.9*	77.1*	76.8	77.4*	81.7*	81.9*
10	d	76.3	75.4*	71.7**	75.2*	71.5	73.1	72.9	73.2	75.6*	75.8*
11	s	153.6	151.4	156.7	152.7	155.0	157.4	157.7	155.4	154.8	152.7
12	s	138.8	136.9	136.9	137.3	134.7	136.9	136.8	134.9	139.8	137.9
13	s	200.5	199.8	200.1	198.8	200.0	199.9	200.0	199.9	199.6	199.5
14	t	44.4	35.7	44.2	44.0	35.7	44.2	44.1	35.5	44.3	35.8
15	s	42.2	37.7	42.4	42.9	37.5	42.4	42.3	44.6	41.2	38.3
16	q	34.1	37.5	34.4	34.0	38.0	34.4	34.3	37.8	34.5	38.0
17	q	20.4	25.7	20.0	20.1	25.4	20.2	20.0	25.2	19.9	24.4
18	q	13.7	13.9	13.9	13.9	14.0	13.9	13.6	13.7	14.1	14.2
19	q	17.8	17.7	17.7	17.6	17.6	17.7	17.6	17.6	17.4	17.4
20	t	118.2	117.8	118.5	116.9	118.4	117.7	117.5	117.2	117.5	117.1
1′	s	170.6	171.2***[d]	171.5***	170.9***	171.1***	171.1	166.2	166.2	166.3	166.2
2′	t	37.4	38.1	38.0	38.3	38.2	38.1	117.5(d)	117.6(d)	117.6(d)	117.5(d)
3′	d	66.0	66.2	66.1	66.3	66.2	66.1	145.6	145.5	145.8	145.7
i-Ph	s	138.8	138.3	138.1	139.6	138.4	138.4	134.2	134.3	134.4	134.4
o-Ph	2d	129.0	128.6	128.7	128.6	128.6	128.6	128.7	128.7	128.9	128.9
m-Ph	2d	128.3	128.0	128.1	128.1	128.0	128.0	128.2	128.2	128.4	128.4
p-Ph	d	128.1	127.4	127.6	127.5	127.4	127.4	130.2	130.1	130.4	130.4
$N(CH_3)_2$	2q	41.7	42.2	42.0	42.2	42.2	42.1				
O_2CMe	s	170.1	170.2***	171.0***	171.7***	171.6***					
					170.0***						
$O_2C\mathit{Me}$	q	21.2	21.2	21.0	21.1	21.0					
					21.1						
$C(CH_3)_2$	d									108.3	108.0
$C(CH_3)_2$	2q									26.8	26.8
										27.2	27.2

[a] Unless indicated differently. [b] Assigment C16-C17 interchanged.[8] [c] Assigment C9-C10 and C16-C17 interchanged.[16] [d] Assigments with the same numbers of asterisks may be interchanged.

Another variable might be the age of the needles. For a rapid screening of the suitability of the taxine mix of different trees and/or varieties as a synthetic starting material without prior hydrolysis and deamination, it is necessary to know which peaks in the taxine chromatogram belong to taxine B and its isomers and which peaks belong to the 1-deoxytaxine B isomer(s). Thus, it was decided to investigate the taxine fraction of *T. baccata* more closely.

In all the taxine samples from the different trees that were analyzed, the same six taxine peaks were found and the largest peaks were always peaks 1 and 3. The minor peaks, 2 and 5, and to a lesser extent also the peaks 4 and 6, were found to be quite variable (see Figure 1 for peak assignment). The major taxines **1** and **3** could be separated from the minor taxines **2**, **4**, **5**, and **6** by column chromatography over neutral alumina (activity grade I) with EtOAc. The taxines **1** and **3** could then be separated by means of prep. TLC on Si gel with CH_2Cl_2:MeOH (20:1). Peak 1 was found to correspond with taxine B (**1**) (for NMR data, see Tables 1 and 2). Both taxine B (**1**) and taxine **3** could be transformed into the same compound **11** by deamination and acetate hydrolysis, which in fact suggested that they were isomers. Furthermore, it was found that both **1** (taxine B) and **3** in their pure forms easily isomerized to the original mixture of **1** and **3**, for example, when kept on a TLC plate overnight. This isomerization is caused by acetyl migrations between C9 and C10. It is a relatively fast process, and also some isomerization was, for instance, found during the recording of ^{13}C-NMR spectra.

The absence and presence of an acetyl group in taxine **3** at C10 and C9, respectively, could also be concluded from its NMR spectra. In the ^{13}C-NMR spectrum of taxine B (**1**) the chemical shift difference between C11 and C12 is 14.8 ppm, while for taxine **3** a chemical shift difference of 19.8 ppm was observed. This leads to the conclusion that the environment of the C11−C12 double bonds of **1** and **3** is chemically different. For compound **11**, in which the alcohol at C10 is likewise not acetylated, the chemical shift difference between C11 and C12 is 20.9 ppm, while in the triacetate **13**, with C10 acetylated, a chemical shift difference of 12.5 ppm was observed.[21] Comparable chemical shift differences have been published.[8,24] A chemical shift difference between C11 and C12 of ca. 20 ppm indicates that the alcohol at C10 is not acetylated, whereas a chemical shift difference of ca. 12−15 ppm proves the presence of an acetate at C10. This observation is only valid for systems with a carbonyl at C13.

The ^{1}H-NMR spectrum of taxine **3** shows a doublet at 5.01 ppm for the allylic proton at C10, which is comparable to the H10 signal at 4.88 ppm in structure **7**. On the other hand, the doublet for H9 in **3** has shifted almost 1.5 ppm to lower field when compared to taxine B (**1**) (5.74 vs. 4.28 ppm). From this, the presence of an acetate at C9 in taxine **3** can also be concluded. It should be noted that the ^{1}H-NMR chemi-

cal shifts for H2 in taxine B (**1**) (3.95 ppm) and its isomer **3** (4.05 ppm) are almost identical, thereby proving that no acetate is present at the C2 position of taxine **3**. The presence of the acetate at C2 in **3** is also precluded by the fact that **3** easily isomerized to **1**, and vice versa. An acetyl migration across the B ring between C2 and C10 is highly improbable.

The minor taxines **2**, **4**, **5**, and **6** were then separated by flash chromatography over Si gel with CH_2Cl_2:MeOH (30:1), to give a mixture (ca. 3:1) of the taxines **4** and **6** and a mixture (ca. 2:1) of the taxines **2** and **5**. Further separation of the mixtures over Si gel or alumina was not possible. The only way to separate the mixtures **4**/**6** and **2**/**5** was by means of preparative RP-HPLC (see Experimental Section). However, we were not successful in isolating the different individual taxines from the buffer solutions sufficiently pure and in sufficient quantities for ^{13}C-NMR spectroscopy. The reason for this was the fact that under neutral or basic conditions, necessary for extracting the taxines from the buffer solution, a very fast isomerization to the original equilibrium mixtures occurred; that is, both pure **2** and **5** almost instantaneously reverted to a mixture of **2** and **5**, and pure **4** reverted to a mixture of **4** and **6**. Compound **6** was never isolated in pure form at any time. Volatile buffers based on ammonium salts were also tried to circumvent the workup step. Although **2**, **5**, and **4**, which were isolated with this procedure looked pure on HPLC, no definitive ^{13}C- and ^{1}H-NMR spectra could be recorded.

For the taxines **2** and **5**, it was, nevertheless, possible to assign the different peaks in the ^{13}C-NMR spectrum of 35 mg of a 2:1 mixture. It clearly showed that **2** and **5** were 1-deoxytaxines: two doublets for C1 at 51.4 and 51.1 ppm, respectively, were observed, while no singlet for C1 was found in the alcohol region between 65 and 80 ppm. Furthermore, the chemical shift differences between C11 and C12 were 14.5 and 20.3 ppm for taxine **2** and taxine **5**, respectively, which is comparable to the differences found in the taxines **1** and **3**. Together with the fact that deamination and deacetylation of the mixture was found to lead to the single compound **12**, it was concluded that the structures were as assigned.

For the taxines **4** and **6** only 8 mg of a 3:1 mixture was isolated, and it was not possible to assign or observe all the peaks in the ^{13}C-NMR spectrum belonging to **6**. It was clear that taxine **4** contained two acetates. Furthermore, the chemical shift difference between C11 and C12 was 15.4 ppm in taxine **4**, which indicated the presence of an acetate at C10 in this compound. As acetate isomerization takes place only between C9 and C10, the decomposition of **4** into **4** and **6** indicated that the second acetate must be at C2. Moreover, it was evident from the ^{13}C-NMR spectrum that **4** possessed a hydroxyl group at C1, which was confirmed by the fact that deamination and deacetylation of the mixture of **4** and **6** resulted in the formation of the single parent compound **11**. On the basis of the above evidence, structures **4** and tentatively **6** for these minor taxines are proposed. The possibility that **6** (t_R 18.5 min) might be identical with 2,9-diacetyltaxine B (**8**) was excluded on basis of the fact that exhaustive acetylation of taxine B (**1**) gave rise to a peak with retention time 24.2 min.

The crude taxine fraction consists for about 40% of compounds possessing a taxane skeleton. For the most part, this 40% is made up by six taxine B derivatives. This observation fits that of Appendino *et al.*, who have stated that the major constituent of taxine is a mixture of at least six taxine-B-related compounds, of which taxine B is the major alkaloid.[14] Our findings are also in agreement with the remark of Poupat *et al.* that isotaxine B (9-acetyl-10-deacetyltaxine B) (**3**) is, together with taxine B, the major alkaloid of the purified taxine fraction.[15] To our knowledge the structures **2**, **4**, **5**, and **6** have not been reported before.

The only other major peak in a freshly prepared crude taxine fraction with a taxine-like UV spectrum was the one around 10 min (see Figure 1). The compound corresponding with this peak was isolated and could be identified as 10-deacetyltaxine B (**7**). This compound is an artifact formed by a partial hydrolysis of taxine B (**1**) during the 4-day extraction of needles with diluted H_2SO_4. When taxine B is kept in diluted H_2SO_4 for 2 days, 10-deacetyltaxine B is formed.

The separation of the taxicin-I and taxicin-II isomers in their *native* alkaloidal form is rather troublesome and would preclude any synthesis of 1-deoxytaxol- or 1,7-dideoxytaxol derivatives. It was found, however, that compounds **11** and **12**, which result after deamination and deacetylation of the initial six taxine B isomers, could be easily converted to their 9,10-monoacetonides **15** and **16**. These corresponding monoacetonides could be well separated with TLC on neutral alumina (R_f values of 0.17 and 0.32 for the monoacetonide with and without the hydroxyl at C1, respectively) or column chromatography on alumina from Woelm or Merck deactivated with 5% water. On alumina from Baker, the acetonides were found to decompose for some reason. As formation of the monoacetonide is part of our synthetic route toward 7-deoxytaxol,[18] this seems to be a good point to separate the two parent skeletons.

Experimental Section

General Experimental Procedures. Chemicals and solvents were of analytical grade, HPLC grade, or distilled prior to use. NMR spectra were recorded on a Bruker AC-E 200 or a Bruker AM 400 spectrometer. MS were recorded on a Finnigan MAT 95 spectrometer. TLC was carried out on ready-made Si gel or alumina plates from Merck containing a fluorescent indicator. Detection was done by viewing under UV 254 nm.

Plant Material. One- to two-year branches of *Taxus baccata* L. were obtained from plants growing on the premises of the Forestry Department, Wageningen Agricultural University, The Netherlands and are identified by Hi-S/946. After being dried for 2−3 h at 60 °C, the needles were separated from the branches and kept in air tight containers until use.

Extraction and Isolation. Dried needles of *T. baccata* (500 g) were soaked in 0.5% (v/v) H_2SO_4 (1 L) with occasional stirring for 4 days. The extract was separated from the needles and extracted with five 300-mL portions of Et_2O to remove the major part of the nondesired neutral organic compounds. Subsequently, the acid solution was brought to pH 10−10.5 by addition of 25% aqueous NH_3. The solution was then extracted with four 300-mL portions of $CHCl_3$, and the combined organic layers were dried (Na_2SO_4), filtered, and evaporated under reduced pressure to yield 3−4.5 g of crude taxine.[17,20]

Table 3. Ternary Gradient System Used for the HPLC Analysis of Taxine

time	MeCN	buffer	MeOH	gradient
0	15	80	5	
13	15	60	25	linear
23	40	35	25	linear
28	60	40	0	linear
30	15	80	5	Waters curve no. 8
35	15	80	5	

Isolation of the Taxines. The minor taxines **2**, **4**, **5**, and **6** were separated from the major taxines **1** and **3** by column chromatography over neutral alumina (activity grade I) with EtOAc as eluent. Both the taxines **1** and **3** were then isolated by means of preparative TLC on Si gel with CH_2Cl_2:MeOH (20:1) as solvent. The fraction containing the minor taxines **2**, **4**, **5**, and **6** was flash chromatographed over Si gel with CH_2Cl_2:MeOH (30:1) to give a pure isomeric mixture of the taxines **4** and **6** and a pure isomeric mixture of the taxines **2** and **5**. Further attempts to separate and isolate the isomeric taxines failed (see Results and Discussion).

HPLC. The apparatus consisted of a Waters 600E multisolvent delivery system, equipped with a Waters 994 programmable photodiode array detector. Data were processed using Waters 991 PDA software. For analyses a 5-μm Rainin Microsorb C18 column (150 × 4.6 mm i.d.) with a 15-mm 5-μm Rainin Microsorb C18 guard column was used. The eluent was a ternary gradient of 0.05 M sodium dihydrogen phosphate solution (7.8 g/L $NaH_2PO_4 \cdot 2H_2O$, pH adjusted to 3.0 with H_3PO_4)−MeCN−MeOH at a flow rate of 2 mL/min. The gradient is given in Table 3. Peaks were detected between 210 and 320 nm, but most chromatograms were plotted at 280 nm.

For preparative HPLC a 250 × 10 mm i.d., 5-μm Rainin Microsorb C18 column was used with different 0.05 M phosphate buffer (pH 3.0)−MeCN or 0.05 M phosphate buffer (pH 3.0)−MeOH gradients as eluent.

Synthesis. 2,9-Diacetyl taxine B (8) was prepared according to Graf.[6] ^{1}H-NMR: see Table 1. CIMS *m/z* (rel. int.) 668 ([M + H]$^+$, 24), 650 (4), 608 (10), 415 (8), 194 (26), 134 (100), 61 (35).

Preparation of *O*-Cinnamoyltaxicin-I (11) and *O*-Cinnamoyltaxicin-II (12) from the Crude Taxine Mixture. To a stirred solution of 1.0 g of crude taxine in 10 mL of anhydrous Et_2O was added 1.0 mL of MeI. The mixture was stirred for 21 h, after which the crystalline MeI adducts of the taxines were filtered off and washed with 25 mL of anhydrous Et_2O. The yellowish crystals were taken into 25 mL of absolute EtOH, and 1.14 g (8.26 mmol) of K_2CO_3 in 25 mL of H_2O was added. The mixture was stirred for 4.5 h at room temperature, and after evaporation of the EtOH, 25 mL of 0.5% H_2SO_4 and 100 mL of brine were added. The aqueous layer was extracted with four 100-mL portions of $CHCl_3$. The combined organic layers were dried (Na_2SO_4) and evaporated under reduced pressure to obtain 0.34 g of a mixture in which the compounds **11** and **12** were the major products in a ratio of ca. 3:1 according to HPLC.

Next, 40 mg of *O*-cinnamoyltaxicin-II (**12**) and 43 mg of *O*-cinnamoyltaxicin-I (**11**), both of them pure according to HPLC analysis, were isolated by means of preparative TLC on alumina with petroleum ether 40−60°:EtOAc (2:1 as solvent (three successive elutions).

Compound 11: ^{1}H-NMR and ^{13}C-NMR see Tables 1 and 2; EIMS *m/z* (rel int) 496 ([M]$^+$, 100), 478 (26), 385 (9), 331 (10), 108 (14); calcd for $C_{29}H_{34}O_6$ *m/z* (M − H_2O) 478.2355, found *m/z* 478.2364.

Compound 12: ^{1}H-NMR and ^{13}C-NMR see Tables 1 and 2; EIMS *m/z* (rel int) 480 ([M]$^+$, 100), 428 (8), 414 (18), 332 (18), 148 (9).

Acetylation of 11 and 12. To a solution of 13.9 mg (0.03 mmol) of **12** in 2 mL of anhydrous pyridine were added 5 drops of Ac_2O and two crystals of DMAP. The reaction mixture was stirred at room temperature for 48 h and then taken into 70 mL of 0.5% H_2SO_4 and extracted with five 20-mL portions of EtOAc. The combined organic layers were washed with 25 mL of brine, dried (Na_2SO_4), and evaporated under reduced pressure. The crude product was chromatographed over neutral alumina (activity grade II) with petroleum ether 40−60°:EtOAc (2:1) to give 15 mg of the triacetate **14**. In a similar fashion **11** was acetylated to the corresponding triacetate **13**. The ^{1}H-NMR data (Table 1) of **13** and **14** were in full agreement with those published.[21]

Preparation of 9,10-Acetonides 15 and 16. A solution of 613 mg of crude mixture of **11** and **12** in 60 mL of anhydrous Me_2CO was stirred for 24 h at room temperature with 3.35 g anhydrous $CuSO_4$ and a few crystals of pTsOH. Next the reaction mixture was filtered over hyflo, evaporated, taken into 200 mL of CH_2Cl_2, and washed with 75 mL of a saturated $NaHCO_3$ solution. Drying, filtration, and evaporation under reduced pressure yielded 584 mg (86%) of the 9,10-isopropylidene derivatives **15** and **16**. Part of the acetonide mixture was purified by means of column chromatography on Woelm alumina (activity grade I) with EtOAc:petroleum ether 40−60°, giving, in order of elution, 110 mg of not completely pure acetonide **16** and 300 mg of pure acetonide **15**. Acetonide **16** was further purified by means of semipreparative HPLC on a Rainin Microsorb C18 column (dimensions 250 × 10 mm) with MeCN:H_2O (7:3) at 4 mL/min as solvent to give 35 mg of the pure acetonide.

Compound 15: ^{1}H-NMR and ^{13}C-NMR see Tables 1 and 2; EIMS *m/z* (rel int) 536 ([M]$^+$, 3), 478 (1), 460 (1), 388 (8), 148 (10), 131 (57); calcd for $C_{32}H_{40}O_7$ *m/z* 536.2774, found *m/z* 536.2780.

Compound 16: ^{1}H-NMR and ^{13}C-NMR see Tables 1 and 2; EIMS *m/z* (rel int) 520 ([M]$^+$, 4), 372 (3), 357 (4), 149 (7), 131 (1); calcd for $C_{32}H_{40}O_6$ *m/z* 520.2825, found *m/z* 520.2824.

Acknowledgment. We are grateful to Pharmachemie B. V., Haarlem, The Netherlands, for financial support of this work, which is part of a collaborative research program between the Departments of Organic Chemistry of Nijmegen University and Wageningen Agricultural University. Further, we wish to thank Mr. A. van Veldhuizen for recording the NMR spectra and Mr. C. J. Teunis for the mass spectral measurements.

References and Notes

(1) Wani, M. C.; Taylor, H. L.; Wall, M. E.; Coggon, P.; McPhail, A. T. *J. Am. Chem. Soc.* **1971**, *93*, 2325−2327.
(2) Lenaz, L.; de Furia, M. D. *Fitoterapia* **1993**, *64*, 27−35.
(3) Schiff, P. B.; Fant, J.; Horwitz, S. B. *Nature* **1979**, *277*, 665−667.
(4) Lucas, H. *Arch. Pharm.* **1856**, *85*, 145−149.
(5) Appendino, G.; Cravotto, G.; Gariboldi, P.; Gabetta, B.; Bombardelli, E. *Gazz. Chim. Ital.* **1994**, *124*, 1−4.

(6) Graf, E. *Arch. Pharm.* **1958**, *291*, 443−449.
(7) Graf, E.; Weinandy, S.; Koch, B.; Breitmaier, E. *Liebigs Ann. Chem.* **1986**, 1147−1151.
(8) Ettouati, L.; Ahond, A.; Poupat, C.; Potier, P. *J. Nat. Prod.* **1991**, *54*, 1455−1458.
(9) Appendino, G.; Tagliapietra, S.; Özen, H. C.; Gariboldi, P.; Gabetta, B.; Bombardelli, E. *J. Nat. Prod.* **1993**, *56*, 514−520.
(10) Graf, E.; Kirfel, A.; Wolff, G.-J.; Breitmaier, E. *Liebigs Ann. Chem.* **1982**, 376−381.
(11) Graf, E.; Bertholdt, H. *Pharm. Zentralhalle Dtschl.* **1957**, *96*, 385−395.
(12) Appendino, G.; Özen, H. C.; Fenoglio, I.; Gariboldi, P.; Gabetta, B.; Bombardelli, E. *Phytochemistry* **1993**, *33*, 1521−1523.
(13) Appendino, G.; Gariboldi, P.; Gabetta, B.; Bombardelli, E. *Fitoterapia* **1993**, *64* (S 1), 37−46.
(14) Appendino, G.; Gariboldi, P.; Gabetta, B.; Bombardelli, E. International Research Congress on Natural Products, Halifax, Canada, 1994, Poster 222.
(15) Poupat, C.; Ahond, A.; Potier, P. *J. Nat. Prod.* **1994**, *57*, 1468−1469.
(16) Ettouati, L.; Ahond, A.; Poupat, C.; Potier, P. *Tetrahedron*. **1991**, *47*, 9823−9838.
(17) van Rozendaal, H. L. M. Some Approaches to the Synthesis of Taxol and Its Derivatives; Total-Synthesis Based on a LHASA Analysis and Semi-Synthesis Starting from Taxine B. Ph.D. Thesis, Katholieke Universiteit, Nijmegen, 1994.
(18) Wiegerinck, P. H. G. to be published.
(19) Kingston, D. G. I.; Molinero, A. A.; Rimoldi, J. M. *Prog. Chem. Org, Nat. Prod.* **1993**, *61*, 1−206.
(20) Baxter, J. N.; Lythgoe, B.; Scales, B.; Scrowton, R. M.; Trippett, S. *J. Chem. Soc.* **1962**, 2964−2971.
(21) Appendino, G.; Gariboldi, P.; Pisetta, A.; Bombardelli, E.; Gabetta, B. *Phytochemistry* **1992**, *31*, 4253−4257.
(22) *Taxane Anticancer Agents: Basic Science and Current Status*; Georg, G. I.; Chen, T. T.; Ojima, I.; Vyas, D. M., Eds.; American Chemical Society: Washington, DC, 1995; Vol. 583.
(23) ElSohly, H. N.; Croom, E. M., Jr.; Kopycki, W. J.; Joshi, A. S.; ElSohly, M. A.; McChesney, J. D. *Phytochem. Anal.* **1995**, *6*, 149−156.
(24) Chmurny, G. N.; Paukstelis, J. V.; Alvarado, A. B.; McGuire, M. T.; Snader, K. M.; Muschik, G. M.; Hilton, B. D. *Phytochemistry* **1993**, *34*, 477−483.

NP960033L