J Wood Sci (2003) 49:548–552
DOI 10.1007/s10086-002-0512-8

NOTE

Nobuyasu Hanari · Hiroshi Yamamoto · Ken-ichi Kuroda

Distinction of resin compounds between the healthy bark and the resinous stem canker of *Thujopsis dolabrata* var. *hondae*

Received: June 3, 2002 / Accepted: December 25, 2002

Abstract Methyl esters of higher fatty acids were detected from the healthy bark of *Thujopsis dolabrata* var. *hondae* but not from the bark of the resinous stem canker of *T. dolabrata* var. *hondae*. This difference enabled us to distinguish healthy trees from diseased ones. Fourteen diterpenes were also isolated from the *n*-hexane extracts of the bark-glued resin taken from the resinous stem canker of *T. dolabrata* var. *hondae*. Of these diterpenes, abietane diterpenes [abieta-7,13-diene (**1**), abietinol (**2**), dehydroabietinol (**4**)], pimarane diterpene [sandaracopimaric acid (**8**)], and labdane diterpenes [manool (**10**), torulosol (**11**), torulosal (**12**), cupressic acid (**13**)] were first isolated from *T. dolabrata* var. *hondae*.

Key words *Thujopsis dolabrata* var. *hondae* · Resinous stem canker · Methyl esters of higher fatty acids · Diterpenes

Introduction

The resinous stem canker of *Chamaecyparis obtusa* is a well-known tree disease that nowadays occurs all over Japan with increasing afforestation areas of *C. obtusa*. Many reports[1–3] on the resinous stem canker of *C. obtusa* are available from the 1920s. Characteristics of this disease are that (1) the resin escapes constantly from the trunk, (2) the cambium is partially necrotized, and (3) the trunk is dented or flattened by nonuniform and hypertrophic growth. Numerous traumatic resin canals are formed in the secondary phloem of the resinous stem canker,[4] lowering the quality as raw materials. A fungal infection is believed to result in such canals, although harmful insects and pressure due to snow weight previously were thought to induce the formation of the canals. Recent studies on resinous stem canker have shown that *Cistella japonica* induces this disease, as resinous lesions similar to those of the resinous stem canker result after inoculating *C. japonica*.[5,6]

Our attention has been drawn to resin flow symptoms associated with the resinous stem canker of *C. obtusa*.[7,8] Qualitative and quantitative research on flowing resin helps clarify the resinous development of resinous stem canker because the defense reactions relate directly to controlling the quality and quantity of flowing resin. Available literature, however, contains few discussions from the point of such view of flowing resin with the resinous stem canker of *C. obtusa*.[9] As a way of understanding resinous stem canker, we examined resin compounds that escaped by traumatic resin canals in the resinous stem canker of *C. obtusa*. We have clarified that (1) many diterpenes, particularly diverse labdane-type diterpenes soluble in *n*-hexane and ethyl acetate extracts, greatly contribute to the constituents of the resin of the resinous stem canker in *C. obtusa*[7,8]; and (2) a diterpene dimer, a characteristic constituent in the healthy bark of *C. obtusa*, is indicative of *C. obtusa* not being infected with the resinous stem canker.[10]

Thujopsis dolabrata var. *hondae* contracts a similar resinous stem canker, a disease that was reported first during the 1980s.[11,12] A few reports[13–15] of damage analysis, seasonal variation in flow resin, and scanning electron microscopic studies on the resinous stem canker of *T. dolabrata* var. *hondae* are available, including some on the pathology of the disease. We therefore examined compounds in the flow resin due to resinous stem canker of *T. dolabrata* var. *hondae*.

In a previous report,[16] we compared terpenes in extracts from the resinous stem canker of *C. obtusa* with those from *T. dolabrata* var. *hondae*. The object of the present study

N. Hanari (✉)
National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba 305-8569, Japan
Tel.+81-29-861-8335; Fax +81-29-861-8335
e-mail: hanari-n@aist.go.jp

H. Yamamoto
Faculty of Education, Ibaraki University, Mito 310-8512, Japan

K. Kuroda
Institute of Agricultural and Forest Engineering, University of Tsukuba, Tsukuba 305-8572, Japan

Part of this paper was presented at the 47th Annual Meeting of the Japan Wood Research Society, Tokyo, April 1999

was to distinguish healthy trees from diseased ones and to clarify the *n*-hexane-soluble compounds involved in the resinous stem canker of *T. dolabrata* var. *hondae*. The results are instructive regarding a better understanding of the disease in *C. obtusa* and *T. dolabrata* var. *hondae*.

Experiment

The analytical apparatuses were similar to those previously reported,[16] except for the eluent of high-performance liquid chromatography (HPLC). The condition of the eluent is described below. Extraction of terpenes from the resinous stem canker of *T. dolabrata* var. *hondae* (kusaate) utilized the same methods previously reported.[16] In addition, healthy *n*-hexane extracts (2.90 g) were obtained from the healthy bark (416.5 g) from *T. dolabrata* var. *hondae* in Tsurugi-Machi, Ishikawa Prefecture.

Gas chromatography-mass spectrometry of *n*-hexane extracts

The following compounds were obtained from the *n*-hexane extracts from the bark-glued resin by gas chromatography-mass spectrometry (GC-MS): Manool (R_t, 5′53″): MS *m/z* (%) 290 (M^+, 2), 272 (27), 137 (100), 81 (98). Abieta-7,13-diene (6′03″): MS *m/z* (%) 272 (M^+, 100), 257 (46), 229 (95), 69 (20). Dehydroabietane (6′06″): MS *m/z* (%) 270 (M^+, 39), 255 (100), 227 (5), 69 (18). Torulosal (8′05″): MS *m/z* (%) 304 (M^+, 1), 289 (3), 286 (22), 275 (1), 81 (100). Totarol (8′47″): MS *m/z* (%) 286 (M^+, 44), 271 (100), 243 (5), 69 (24). Ferruginol (9′02″): MS *m/z* (%) 286 (M^+, 100), 271 (83), 243 (5), 69 (35). *trans*-Communic acid (9′36″): MS *m/z* (%) 302 (M^+, 61), 287 (37), 257 (20), 81 (100). Abietinol (10′09″): MS *m/z* (%) 288 (M^+, 100), 273 (15), 257 (31), 245 (21), 69 (24).

The following compounds were obtained from the *n*-hexane extracts from the healthy bark: Methyl palmitate (4′22″): MS *m/z* (%) 270 (M^+, 17), 239 (5), 227 (9), 74 (100). Methyl stearate (5′32″): MS *m/z* (%) 298 (M^+, 15), 267 (2), 255 (7), 74 (100). Methyl linoleate (5′38″): MS *m/z* (%) 294 (M^+, 24), 263 (10), 251 (1), 81 (93), 67 (100). Methyl linolenate (5′47″): MS *m/z* (%) 292 (M^+, 12), 261 (5), 249 (1), 79 (100), 67 (73). Manool (5′53″). Abieta-7,13-diene (6′02″). Dehydroabietane (6′05″). Methyl eicosanoate (6′41″): MS *m/z* (%) 326 (M^+, 24), 295 (4), 283 (9), 74 (100). Methyl eicosatrienoate (6′47″): MS *m/z* (%) 320 (M^+, 5), 289 (1), 277 (1), 79 (65), 67 (100). Torulosal (8′03″). Methyl docosanoate (8′25″): MS *m/z* (%) 354 (M^+, 37), 323 (4), 311 (9), 74 (100). Totarol (8′52″). Methyl tricosanoate (9′30″): MS *m/z* (%) 368 (M^+, 45), 337 (4), 325 (12), 74 (100). Methyl tetracosanoate (11′08″): MS *m/z* (%) 382 (M^+, 52), 351 (4), 339 (11), 74 (100). Methyl pentacosanoate (13′01″): MS *m/z* (%) 396 (M^+, 45), 365 (2), 353 (10), 74 (100). Methyl hexacosanoate (15′47″): MS *m/z* (%) 410 (M^+, 47), 379 (3), 367 (11), 74 (100).

Isolation of diterpenes from the *n*-hexane extracts

The *n*-hexane extracts (6.03 g) were separated from fraction (Fr.) Fr. 1 to Fr. 4 by silica gel column chromatography (CC, flash, 30 g) using *n*-hexane-ethyl acetate (9:1 v/v) as an eluent (Fig. 1), similar to the separation scheme reported before.[7,8,10,16] Fraction 1 (622.4 mg) was separated from Fr. 11 to Fr. 13 by preparative HPLC (Shim-pack PREP-ODS column; eluent MeOH; flow rate 7.5 ml min^{-1}). Fraction 2 (1.77 g) was separated from Fr. 21 to Fr. 25 by preparative HPLC (Shim-pack PREP-ODS column; eluent MeOH-H_2O 9:1; flow rate 7.5 ml min^{-1}). Fraction 25 (142.5 mg) was separated from Fr. 251 to Fr. 253 by preparative HPLC (ODS-80Ts column; eluent MeOH-H_2O 8:2; flow rate 2.5 ml min^{-1}). Fraction 3 (1.38 g) was separated from Fr. 31 to Fr. 35 by preparative HPLC (Shim-pack PREP-ODS

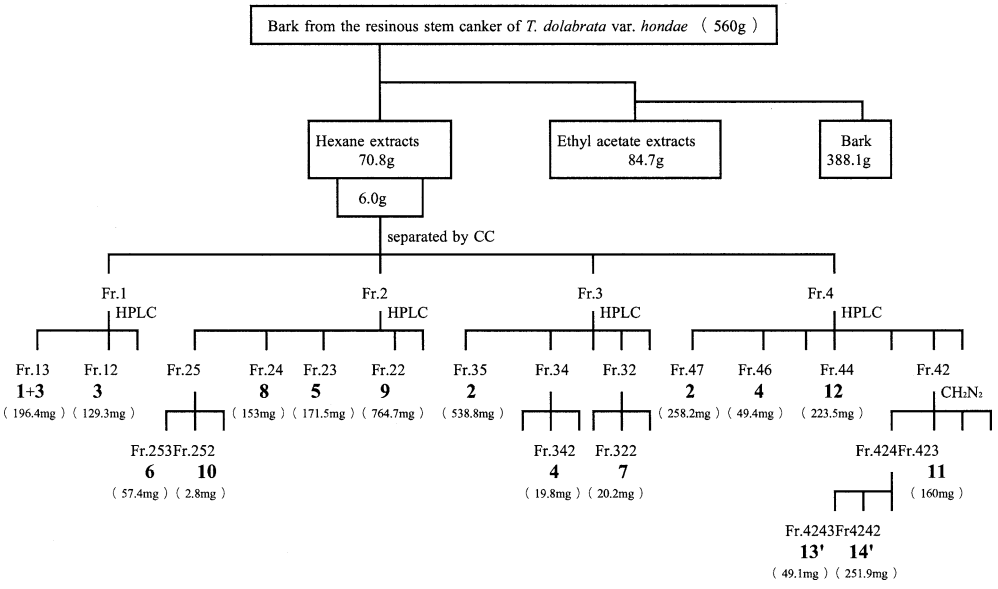

Fig. 1. Separation schema for diterpenes from the bark-glued resin of the resinous stem canker of *Thujopsis dolabrata* var. *hondae*. *Prime* indicates a methyl ester of a related acid. *CC*, silica gel column chromatography; *HPLC*, high performance liquid chromatography

column; eluent MeOH-H_2O 9:1; flow rate 7.5 ml min^{-1}). Fraction 32 (52.2 mg) was separated from Fr. 321 to Fr. 323 by preparative HPLC (ODS-80Ts column; eluent MeOH-H_2O 8:2; flow rate 2.5 ml min^{-1}). Fraction 34 (90.9 mg) was separated from Fr. 341 to Fr. 343 by preparative HPLC (ODS-80Ts column; eluent MeOH-H_2O 8:2; flow rate 2.5 ml min^{-1}). Fraction 4 (1.86 g) was separated from Fr. 41 to Fr. 47 by preparative HPLC (Shim-pack PREP-ODS column; eluent MeOH-H_2O 9:1; flow rate 7.5 ml min^{-1}). Fraction 42 (566.1 mg) was methylated with an ethereal diazomethane solution at room temperature.[17,18] Methylated Fr. 42 (547.8 mg) was separated from Fr. 421 to Fr. 423 by preparative HPLC (ODS-80Ts column; eluent MeOH-H_2O 8:2; flow rate 2.5 ml min^{-1}). Fraction 424 (303.1 mg) was separated from Fr. 4241 to Fr. 4243 by preparative HPLC (ODS-80Ts column; eluent MeOH-H_2O 7:3; flow rate 2.5 ml min^{-1}).

Isolated diterpenes

The following 14 diterpenes were isolated from the *n*-hexane extracts from the resinous stem canker of *T. dolabrata* var. *hondae*. Identification was done by comparing analytical data of isolated diterpenes with published data.

Abieta-7,13-diene (**1**). Fraction 13 (196.4 mg) was identified as mixture of diterpenes **1**[19,20] and **3**[16] (Fig. 2). The relative ratio of **1** and **3** (5:1) were calculated on the basis of the integrated intensity of 14-H signal in the ^{1}H-NMR spectrum. ^{1}H-NMR ($CDCl_3$): δ ppm 0.79 (s, 3H, Me-20), 0.86 (s, 3H, Me-19), 0.91 (s, 3H, Me-18), 1.00 (d, 3H, J = 7.0 Hz, *i*-Pr.), 1.01 (d, 3H, J = 7.0 Hz, *i*-Pr.), 5.42 (br.s, 1H, H-7), 5.78 (s, 1H, H-14).

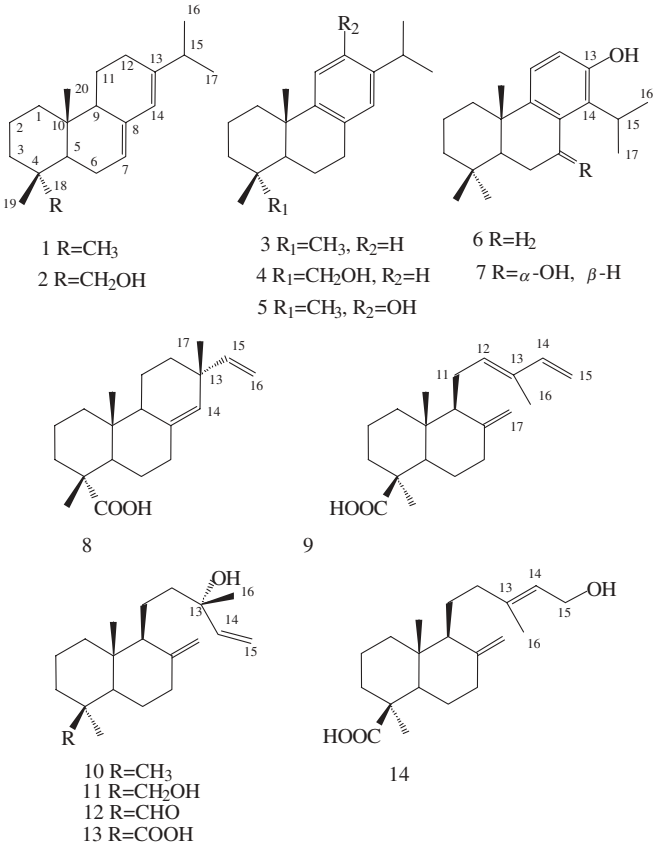

Fig. 2. Structures of abietane (*1–5*), totarane (*6–7*), pimarane (*8*), and labdane (*9–14*) diterpenes

Abietinol; abieta-7,13-dien-18-ol (**2**). Fraction 35 (538.8 mg) and Fr. 47 (258.2 mg) were identified as abietinol by ^{1}H-NMR analysis. Colorless oil (crystal).[21] ^{1}H-NMR ($CDCl_3$): δ ppm 0.83 (s, 3H, Me-20), 0.88 (s, 3H, Me-19), 1.00 (d, 3H, J = 7.0 Hz, *i*-Pr.), 1.01 (d, 3H, J = 7.0 Hz, *i*-Pr.), 2.22 (sept, 1H, J = 7.0 Hz, H-15), 3.13 (d, 1H, J = 11.0 Hz, H-18), 3.36 (d, 1H, J = 11.0 Hz, H-18), 5.39 (br.s, 1H, H-7), 5.77 (s, 1H, H-14); IR ν_{max} (KBr) cm^{-1}: 3422, 1459, 1384, 1041.

Dehydroabietane; abieta-8,11,13-triene (**3**). Fraction 12 (129.3 mg) was identified as dehydroabietane by comparing our previous report.[16]

Dehydroabietinol; abieta-8,11,13-trien-18-ol (**4**). Fractions Fr. 342 (19.8 mg) and Fr. 46 (49.4 mg) were identified as dehydroabietinol by comparing our previous report.[16]

Ferruginol (**5**). Fraction 23 (171.5 mg) was identified as ferruginol by comparing ^{1}H-NMR and MS spectra data with those of authentics.[7,8,10,16] Colorless oil, ^{1}H-NMR ($CDCl_3$): δ ppm 0.91 (s, 3H, Me-20), 0.94 (s, 3H, Me-19), 1.17 (s, 3H, Me-18), 1.22 (d, 3H, J = 7.0 Hz, *i*-Pr.), 1.24 (d, 3H, J = 7.0 Hz, *i*-Pr.), 3.11 (sept, 1H, J = 7.0 Hz, H-15), 6.63 (s, 1H, H-11), 6.83 (s, 1H, H-14); MS: *m/z* (%) 286 (M$^+$, 100), 271 (85), 243 (2), 69 (39).

Totarol (**6**). Fraction 253 (57.4 mg) was identified as totarol by comparing our previous report.[16]

7α-Hydroxytotarol (**7**). Fraction 322 (20.2 mg) was identified as 7α-hydroxytotarol by comparing our previous report.[16]

Sandaracopimaric acid (**8**). Fraction 24 (153 mg) was identified as sandaracopimaric acid by comparing 1H-NMR and MS spectra data with those of authentics.[10] Crystal, ^{1}H-NMR (DMSO-d_3): δ ppm 0.79 (s, 3H, Me-20), 1.01 (s, 3H, Me-17), 1.10 (s, 3H, Me-19), 2.17 (s, 1H, H-9), 4.86 (d, 1H, J = 11.0 Hz, H-16), 4.88 (d, 1H, J = 17.6 Hz, H-16), 5.19 (s, 1H, H-14), 5.73 (dd, 1H, J = 11.0 and 17.6 Hz, H-15), 11.99 (br.s, 1H, COOH-18); MS: *m/z* (%) 302 (M$^+$, 76), 287 (66), 257 (16), 121 (100).

trans-Communic acid; 8 (17), 12,14-labdatrien-19-oic acid (**9**). Fraction 22 (764.7 mg) was identified as *trans*-communic acid by comparing our previous reports.[7,8,10,16]

Manool; 8 (17), 14-labdadien-13*R*-ol (**10**). Fraction 252 (2.8 mg) was identified as manool by comparing our previous reports.[7,8,16]

Torulosol; 8 (17), 14-labdadien-13*R*, 19-diol (**11**). Fraction 423 (160 mg) was identified as torulosol by comparing ^{1}H-NMR and MS spectra data with those of authentics.[7,10,16] Colorless oil, ^{1}H-NMR ($CDCl_3$): δ ppm 0.64 (s, 3H, Me-20), 0.98 (s, 3H, Me-18), 1.27 (s, 3H, Me-16), 3.38 (d, 1H, J = 10.9 Hz, H-19), 3.75 (d, 1H, J = 10.9 Hz, H-19), 4.48 (s, 1H, H-17), 4.80 (s, 1H, H-17), 5.06 (d, 1H, J = 10.9 Hz, H-15), 5.21 (d, 1H, J = 17.5 Hz, H-15), 5.91 (dd, 1H, J = 10.9 and 17.5 Hz, H-14); MS: *m/z* (%) 306 (M^+, 1), 291 (2), 288 (7), 275 (5), 81 (100).

Torulosal; 13*R*-hydroxy-8 (17), 14-labdadien-19-al (**12**). Fraction 44 (223.5 mg) was identified as torulosal by comparing our previous reports.[7,16]

Methyl cupressate; methyl 8 (17), 14-labdadien-19-oate (**13′**). Fraction 4243 (49.1 mg) was identified as methyl cupressate by comparing ^{1}H-NMR and MS spectra data with those of authentics.[10,16] Colorless oil, ^{1}H-NMR ($CDCl_3$): δ ppm 0.50 (s, 3H, Me-20), 1.18 (s, 3H, Me-18), 1.27 (s, 3H, Me-16), 3.61 (s, 3H, OMe), 4.48 (s, 1H, H-17), 4.83 (s, 1H, H-17), 5.06 (dd, 1H, J = 1.0 and 10.6 Hz, H-15), 5.20 (d, 1H, J = 17.5 Hz, H-15), 5.91 (dd, 1H, J = 10.6 and 17.5 Hz, H-14); MS: *m/z* (%) 334 (M^+, 3), 316 (27), 301 (24), 274 (7), 121 (100), 81 (40).

Methyl isocupressate; methyl 15-hydroxy-8 (17), 13-labdadien-19-oate (**14′**). Fraction 4242 (251.9 mg) was identified as methyl isocupressate by comparing ^{1}H-NMR and MS spectra data with those of authentics.[10,22] Colorless oil, ^{1}H-NMR ($CDCl_3$): δ ppm 0.50 (s, 3H, Me-20), 1.18 (s, 3H, Me-18), 1.67 (s, 3H, Me-16), 3.62 (s, 3H, OMe), 4.14 (d, 2H, J = 6.9 Hz, H-15), 4.52 (s, 1H, H-17), 4.85 (s, 1H, H-17), 5.38 (t, 1H, J = 6.9 Hz, H-14); MS: *m/z* (%) 334 (M^+, 1), 316 (7), 301 (6), 274 (2), 121 (100), 81 (26); IR ν_{max} (KBr) cm^{-1}: 1725, 1154, 989, 888.

Results and discussion

The *n*-hexane extracts and ethyl acetate extracts obtained from the bark-glued resin had concentrations of 12.6 g/100 g air-dried bark and 15.1 g/100 g, respectively. They were 18.0 and 3.07 times as heavy as those from the healthy bark, respectively. GC-MS analysis of the resinous stem canker and the healthy bark samples revealed large differences in the ratios and compositions of the diterpenes of the two barks (Fig. 3). We supported the hypothesis that the scatter of analysis data among specimens was not significant in the resinous stem canker samples and the healthy bark samples. These extracts were confirmed diterpenes (**1**, **3**, **6**, **10**, **12**) that are commonly found in both barks. Methyl esters of higher fatty acids (C16–26) were detected only in the healthy bark (Table 1). The total area of selected peaks was normalized at 100%; these esters accounted for 35.3% of the selected GC-MS signal areas. Therefore, methyl esters of higher fatty acids are characteristic compounds of healthy bark from *T. dolabrata* var. *hondae*. A diterpene dimer, 6-(abieta-6′,8′,11′,13′-tetraenyl-12′-oxy)-7-methoxyabieta-8,11,13-trien-12-ol, is a terpene indicative of *C. obtusa* not infected with the resinous stem canker.[8] Therefore, the esters, together with the dimer, are key compounds for distinguishing diseased trees from healthy ones. We are able to distinguish disease trees from healthy ones by the presence of these compounds in the barks.

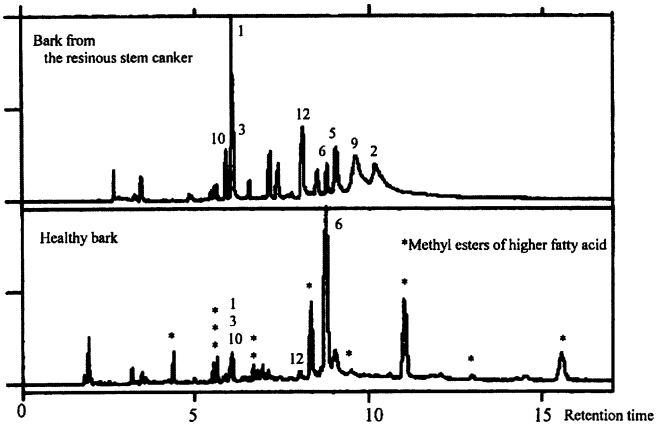

Fig. 3. Total ion monitored gas chromatograms of the *n*-hexane extracts from two bark samples of *Thujopsis dolabrata* var. *hondae*. *Numbers* indicate the number of diterpenes. * Methyl ester of a higher fatty acid

Table 1. Methyl esters of higher fatty acids from the healthy bark of *Thujopsis dolabrata* var. *hondae*

Peak no.	Compound	Retention time (minutes, seconds)	Rational formula
*1	Methyl palmitate	4′22″	$C_{15}H_{31}COOCH_3$
*2	Methyl stearate	5′32″	$C_{17}H_{35}COOCH_3$
*3	Methyl linoleate	5′38″	$C_{17}H_{31}COOCH_3$
*4	Methyl linolenate	5′47″	$C_{17}H_{29}COOCH_3$
*5	Methyl eicosanoate	6′41″	$C_{19}H_{39}COOCH_3$
*6	Methyl eicosatrienoate	6′47″	$C_{19}H_{33}COOCH_3$
*7	Methyl docosanoate	8′25″	$C_{21}H_{43}COOCH_3$
*8	Methyl tricosanoate	9′30″	$C_{22}H_{45}COOCH_3$
*9	Methyl tetracosanoate	11′08″	$C_{23}H_{47}COOCH_3$
*10	Methyl pentacosanoate	13′01″	$C_{24}H_{49}COOCH_3$
*11	Methyl hexacosanoate	15′47″	$C_{25}H_{51}COOCH_3$

Fourteen diterpenes were also isolated from the *n*-hexane extracts of the bark-glued resin of the resinous stem canker of *T. dolabrata* var. *hondae* and were identified by nuclear magnetic resonance (NMR) and MS analyses. Some of the isolated diterpenes were known compounds: **3**,[23] **6**,[24] and **7**[24] from the leaves of *T. dolabrata* Sieb. et Zucc.; **3**, **5**, and **6** from the callus *of T. dolabrata*[25]; **3**, **5**, **6**, and **7** from the seeds of *T. dolabrata* var. *dolabrata*[26]; and **3**, **5**, **6**, **9**, and **14** from the seeds of *T. dolabrata* var. *hondae*.[27] Diterpene **9** was also an effective criterion for the chemotaxonomy of varieties of *Thujopsis* species.[28] Other than these diterpenes, to our knowledge the rest (**1**, **2**, **4**, **8**, and **10–13**) were first isolated from *T. dolabrata* var. *hondae* and *T. dolabrata*. Table 2 shows that diterpene **2** was the most abundant, 13.3% weight, in the *n*-hexane extracts of the diseased *T. dolabrata* var. *hondae* but absent in the *n*-

Table 2. Terpenes identified in healthy bark and the bark of the resinous stem canker of *Thujopsis dolabrata* var. *hondae*

Compound[a]	Detection of *T. dolabrata* var. *hondae*	
	Healthy bark	Resinous stem canker
1	Yes[b]	Yes[b] (++)
2	No	Yes[b] (+++)
3	Yes	Yes (++)
4	No	Yes[b] (++)
5	No	Yes (++)
6	Yes	Yes (+)
7	No	Yes (+)
8	No	Yes[b] (++)
9	No	Yes (+++)
10	Yes[b]	Yes[b] (+)
11	No	Yes[b] (++)
12	Yes[b]	Yes[b] (++)
13	No	Yes[b] (+)
14	No	Yes (++)
Methyl esters of higher fatty acids	Yes	No

Yes, detected; No, not detected; +, ++, +++, yields of <2%, 2%–5%, and >5%, respectively, based on the *n*-hexane
[a] Number of the isolated terpene
[b] First isolated from *T. dolabrata* var. *hondae*

hexane extracts from the healthy bark by GC-MS analysis. This suggests that diterpene **2** is a characteristic compound from the resinous stem canker of *T. dolabrata* var. *hondae*. However, diterpene **9**, abundant (12.8% weight) next to **2**, is not a characteristic compound because it was reported that diterpene **9** was contained in seeds of *T. dolabrata* var. *hondae*. These findings demonstrate that some diterpenes increase with disease, similar to the findings of Westfelt.[29]

Acknowledgments The authors thank Dr. Y. Yada, Ishikawa Forest Experiment Station, for supplying samples (the resinous stem canker and the healthy bark of *T. dolabrata* var. *hondae*). The authors are grateful to the Chemical Analysis Center, University of Tsukuba, for NMR spectra and elemental analysis data.

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