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A new diterpene dimer from the bark of *Chamaecyparis obtusa*

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Abstract This paper describes characterization of diterpenes of the bark of Japanese cypress, *Chamaecyparis obtusa* (S. and Z.) Endl, without the resinous stem canker to learn the difference between the cypress bark affected with the canker. A diterpene dimer and two diterpenes, 6α,12-dihydroxyabieta-8,11,13-trien-7-one and 6,12-dihydroxyabieta-5,8,11,13-tetraen-7-one, were firstly isolated from Japanese cypress. The dimer, 6-(abieta-6′,8′,11′,13′-tetraenyl-12′-oxy)-7-methoxyabieta-8,11,13-trien-12-ol, was a new compound. It is a terpene indicative of *C. obtusa* not infected with the resinous stem canker. Five known diterpenes were also isolated.

Key words Diterpene dimer · Diterpene · *Chamaecyparis obtusa* · Resinous stem canker

Introduction

Japanese cypress is an evergreen that ordinarily grows in the hills from Honshu to Shikoku and Kyushu. It is known for its beautiful grain and fragrance, and it is a high permanent tree. It has been used for shrines and statues of Buddha and as a high-quality building material.

Japanese cypress is often affected by the resinous stem canker. Interest in the canker has been increasing, as resin constantly escapes from the canker lesions, resulting in a stain on the surface of the bark.[1–6] Consequently, the resinous stem canker has decreased the market value of the timber. In cypress with this disease the cambium is partially necrotized,[2] and the trunk is dented or flattened by non-uniform and hypertrophic growth.[3] Anatomical observations from these affected parts have shown that numerous traumatic resin canals are formed in the secondary phloem.[4] They are arranged in tangential series usually with radial intervals.[4,6] Resinous lesions similar to those of the resinous stem canker result from inoculating *Cistella japonica*.[5] Traumatic resin canals start in the ring grown during the current year or the year prior to the inoculation.[6]

We have become aware of the constituents of the resin of the resinous stem canker through the few available reports on this matter.[7–9] The resin of Japanese cypress affected with the resinous stem canker comprises many diterpenes.[7] Previous reports[8,9] on the resinous stem canker of cypress showed the presence of various labdane diterpenes. Research efforts were directed at characterizing the constituents of the extracts, particularly diterpenes, from the barks with and without resinosis of the cypress to clarify the contribution of the extracts to the resinous stem canker of the cypress wood and the diterpenes involved in the resinous development.

The object of this study was to determine the characteristic constituents of the bark without resinosis and the difference between healthy bark and the resinous stem canker from the view of the constituents of extracts of the barks. The results provide a better understanding of the mechanism of the resinous development by the resinous stem canker. The paper forms part of a wider study on the resinous stem canker of Japanese cypress.

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Results and discussion

n-Hexane extracts of the bark without resinosis

The *n*-hexane extracts obtained from the outer bark without resinosis had a concentration of 0.84 g/100 g air-dried bark, corresponding to their relative weight: They were 1/30 as heavy as the resinous stem canker. The characteristic

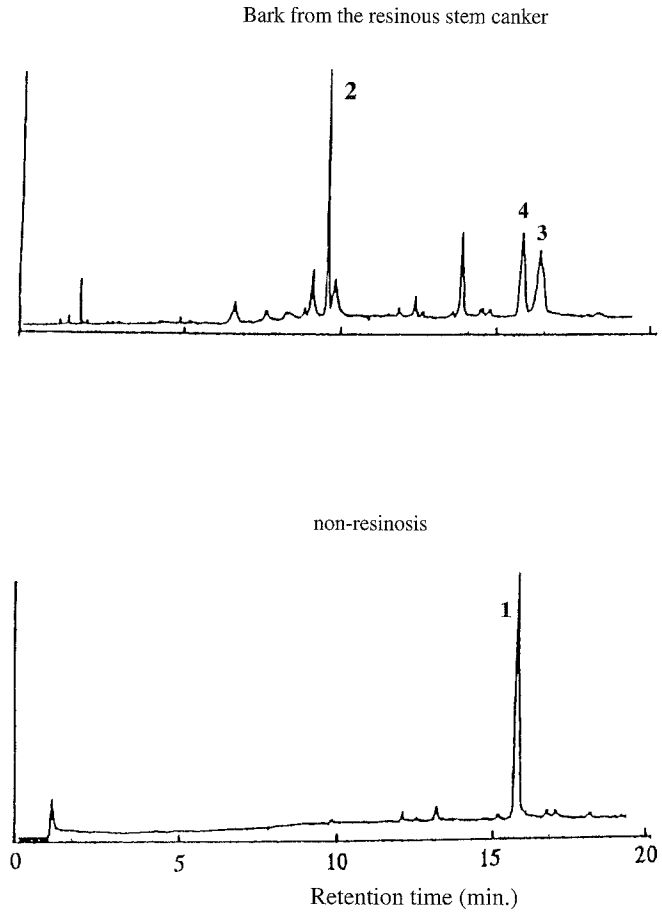


Fig. 1. Total ion monitored gas chromatograms of *n*-hexane extracts from the bark of the resinous stem canker and the non-resinosis. Numbers are the same as those of terpenes

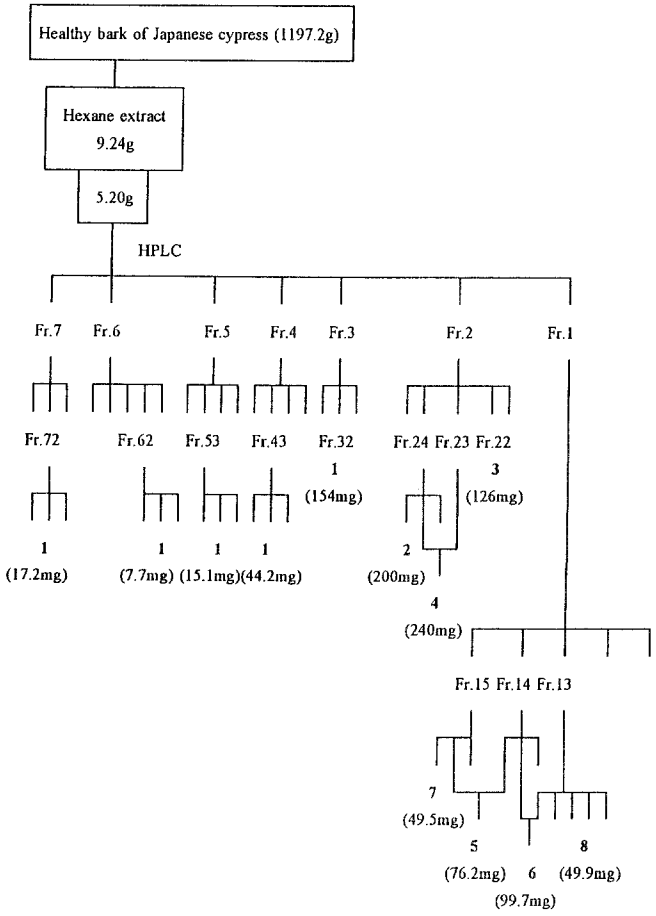


Fig. 2. Separation scheme for terpenes from the bark without resinosis. Compounds **1–8**. See Figs. 3 and 4

constituents were shown by gas chromatography-mass spectrometry (GC-MS) analysis of the *n*-hexane extracts (Fig. 1).

Isolation and identification of terpenes from the *n*-hexane extracts

The *n*-hexane extracts (5.20g) were repeatedly separated by preparative high performance liquid chromatography (HPLC) (Fig. 2). The compounds obtained by the first separation procedure were numbered as Fr. 1, Fr. 2; double figures were used to identify those obtained by the second separation procedure (e.g., Fr. 21, Fr. 22). The compounds obtained by further separation procedures were numbered with three and four figures.

Isolation and structure of 6-(abieta-6′,8′,11′,13′-tetraenyl-12′-oxy)-7-methoxyabieta-8,11,13-trien-12-ol (**1**)

Compound **1** was isolated as a colorless powder in Fr. 32, 432, 532, 622, and 722. The molecular formula $C_{41}H_{58}O_3$ was obtained from the mass measurement. The proton-1 nuclear magnetic resonance (^{1}H-NMR) spectrum contained two isopropyl, six quaternary methyl, one methoxy (δ 3.15ppm), two olefin of Z-configuration (δ 5.90, 6.52ppm), and four aromatic signals. The carbon-13 NMR (^{13}C-NMR) spectrum showed the existence of 41 carbons in compound **1**. With the aid of two-dimensional (2D) NMR techniques, COSY, HSQC, HMBC, and NOESY, all the ^{1}H-NMR and ^{13}C-NMR signals were assigned to the two abietane moieties; one was 6,7-dehydroferruginol and the other 6′-oxy-7′-methoxyferruginol moieties including their relative configuration (Table 1). The combining position of two diterpene moieties, 6C-O-12′ C ether linkage, was determined by the long-range coupling of 6H and 12′ C detected in the HMBC experiment; this was supported by the nuclear overhauser effect (NOE) correlation of 6H-11′ H, 7H-11′ H, 7OMe-11′ H, and 7OMe-1′ eqH. Unfortunately, insufficient amounts of material remained to determine the relative configuration between the two abietane moieties and the absolute configuration (Fig. 3).

Isolation of manool (**2**), *trans*-communic acid (**3**), ferruginol (**4**), and sugiol (**5**)

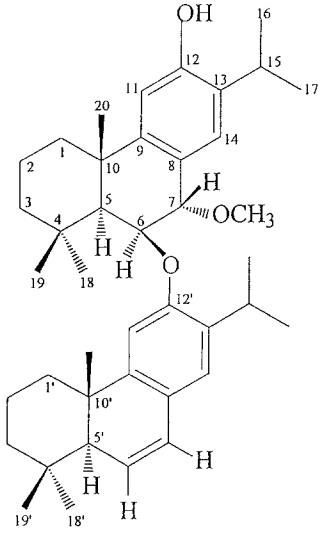
Compound **2** as Fr. 243, compound **3** as Fr. 22, compound **4** as Fr. 23 and 242, and compound **5** as Fr. 143 and 152 were isolated, respectively. Compounds **2**, **3**, **4**, and **5** were identified as manool [8(17),14-labdadien-13*R*-ol], *trans*-

Table 1. ^{13}C and ^{1}H NMR data for **1** in $CDCl_3$[a]

No.	^{13}C	^{1}H (J in Hz)	No.	^{13}C	^{1}H (J in Hz)
1	39.39 (t)	eq2.13brd (12.3) ax1.57brt (12)[b]	1′	36.18 (t)	eq2.24brd (11.4)[b] ax1.71brt (12)[b]
2	18.92 (t)	eq1.67brd (12)[b] ax1.84brq (12)[b]	2′	19.00 (t)	1.76m[b] 1.76m[b]
3	42.74 (t)	eq1.48brd (12)[b] ax1.28brt (12)[b]	3′	41.03 (t)	eq1.54brd (12)[b] ax1.27brt (12)[b]
4	34.19 (s)		4′	32.81 (s)	
5	55.55 (d)	1.65d (8.6)	5′	50.94 (d)	2.17dd (3.0, 2.6)
6	78.27 (d)	4.91d (8.6)	6′	127.34 (d)	5.90dd (9.6, 2.6)
7	81.72 (d)	3.98s	7′	127.17 (d)	6.52dd (9.6, 3.0)
8	124.47 (s)		8′	125.42 (s)	
9	150.67 (s)		9′	146.89 (s)	
10	37.84 (s)		10′	38.07 (s)	
11	110.28 (d)	6.74s	11′	106.54 (d)	7.04s
12	152.97 (s)		12′	152.88 (s)	
13	130.55 (s)		13′	134.77 (s)	
14	129.38 (d)	6.88s	14′	124.55 (d)	6.93s
15	26.44 (d)	3.13sept (6.9)[b]	15′	25.40 (d)	3.18sept (6.9)
16	22.44 (q)	1.19d (6.9)	16′	23.07 (q)	1.05d (6.9)
17	22.58 (q)	1.22d (6.9)	17′	23.09 (q)	1.07d (6.9)
18	35.04 (q)	0.93s	18′	32.54 (q)	0.99s
19	22.81 (q)	1.17s	19′	22.49 (q)	1.08s
20	24.54 (q)	1.39s	20′	20.33 (q)	1.12s
7-OMe	55.00 (q)	3.15s			

brd, broad doublet; brt, broad triplet; brq, broad quartet; eq, equatorial; ax, axial
[a] Signals were assigned on the basis of COSY, HSQC, HMBC, PSNOESY, and difference NOE experiment
[b] Multiplicity and J values were determined by cross peaks of HSQC spectrum

Fig. 3. Structure of compound **1**



communic acid [8(17),12,14-labdatrien-19-oic acid], ferruginol, and sugiol, respectively, by comparing their NMR and MS spectra data with those of authentics[8,9] (Fig. 4).

Isolation of 6α,12-dihydroxyabieta-8,11,13-trien-7-one (**6**), 6,12-dihydroxyabieta-5,8,11,13-tetraen-7-one (**7**), and 12-hydroxy-6,7-secoabieta-8,11,13-trien-6,7-dial (**8**)

Compound **6** as Fr. 135 and Fr. 142, compound **7** as Fr. 153, and compound **8** as Fr. 132 were isolated, respectively. Compounds **6**, **7**, and **8** were identified as 6α,12-dihydroxyabieta-8,11,13-trien-7-one, 6,12-dihydroxyabieta-5,8,11,13-tetraen-7-one, and 12-hydroxy-6,7- secoabieta-8, 11,13-trien-6,7-dial, respectively, by comparing the NMR and MS spectra data with published data.[10–12]

A diterpene dimer and seven diterpenes were isolated from the *n*-hexane extracts of the bark from Japanese cypress. Diterpene dimer **1** was a new compound and is a characteristic constituent from the bark without resinosis. Abietane diterpenes **6** and **7** were first isolated from *C. obtusa*. Terpenes are secondary metabolites in plants. Terpenes with 12 carbons, diterpenes, are known as antimicrobial substance.[13,14] Biosynthetic precursors for tricyclic diterpenes (e.g., abietane and pimarane type) are labdane types of dicyclic diterpenes.[15] All biosynthesis pathways work regularly in healthy bark; therefore many abietane and pimarane diterpenes are produced from labdane diterpenes. Abietane and pimarane diterpenes are widely distributed in conifers. In contrast, traumatic resin canals usually are not present in healthy Japanese cypress[2–4], so little resin is extracted from healthy bark.

In this study, the bark without resinosis contained many abietane-type diterpenes, **1** and **4**–**8**. Abietane compounds were isolated from the *n*-hexane extracts at a yield of 14.5% and labdane compounds at 6.27%. The bark of the resinous stem canker, on the other hand, contained many labdane diterpenes[8–9]; labdane compounds in 15% yield and abietane compounds in 7.6% yield were isolated from the *n*-hexane extracts, respectively.[8] Moreover, the quantity of the *n*-hexane extracts from the bark of the resinous stem canker was 30 times heavier than those from the bark without resinosis.

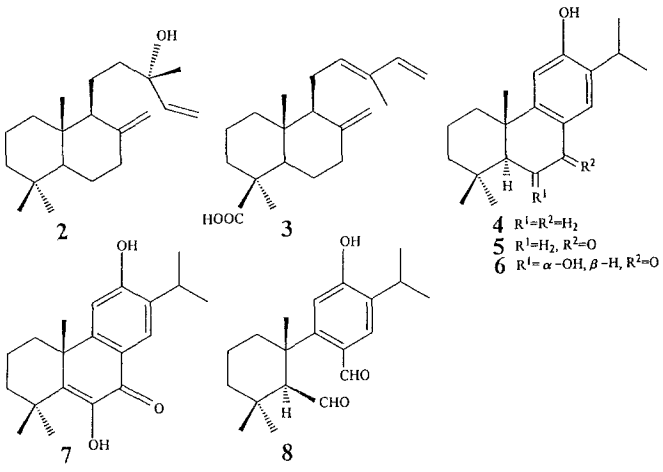


Fig. 4. Structures of diterpenes **2–8**

Infection of trees with this disease increases the early biosynthetic products, labdane diterpenes, compared to that in bark without resinosis. The resin of the bark with the resinous stem canker contained these diterpenes, which constantly escaped from the canker lesions. In addition, numerous traumatic resin canals were formed in the secondary phloem,[4] and resin production was abnormally activated around the affected parts.[4,6] These phenomena may be due to the following: When trees are infected with the resinous stem canker due to penetration of the pathogen, numerous traumatic resin canals are formed to resist the disease. Trees probably defend themselves from further progress of the disease by producing resin that contains antimicrobial terpenes. Moreover, the trees' normal metabolic pathways are stressed by the infections situation, so labdane diterpenes produced earlier are released from the canker lesions.

In future, we must investigate the possibility that constituents of the bark vary according to their lineage and location and the season.

Experiment

Electron impact mass spectra (EIMS) were obtained using a JEOL JMS-DX300 spectrometer at an ionizing voltage of 70eV. GC-MS was performed using an OV-17 capillary column (0.32mm ID × 15m; column temperature 100°–230°C (16°C/min); injector temperature 250°C; carrier gas He 40ml/min). ^{1}H and ^{13}C-NMR spectra were measured with a JEOL GSX-400 spectrometer in $CDCl_3$ and dimethylsulfoxide (DMSO)-*d* 6 with tetramethylsilane as an internal standard. HPLC analysis was performed using a JASCO 880 pump with a SIL C_{18}T column (7.2mm ID × 25cm; eluent MeOH/H_2O 10:0–8:2v/v; flow rate 2.5ml/min) and a JASCO 875-UV detector (λ 230nm). Preparative HPLC was performed using a JASCO instrument with a Shim-pack PREP-ODS column (20mm ID × 25cm; eluent MeOH/H_2O 9:1–8:2v/v or MeOH/CH_2Cl_2 5:1v/v; flow rate 7.5ml/min) or an ODS-80Ts column (column size 7.8mm ID × 30cm; eluent MeOH/CH_2Cl_2/H_2O 10:2:1v/v; flow rate 2.5ml/min).

Extraction of terpenes

The outer bark from *C. obtusa*, a planted 70-year-old tree (diameter 35cm) without resinosis in a grove that existed a few trees of the resinous stem canker, was obtained in Ogawa-Mura, Ibaraki Prefecture (May 10, 1997). Air-dried bark (1197.2g) was crushed to extract in *n*-hexane (8l × 2) for 4 days at room temperature. Evaporation of the solvent left a yellow sticky extract (9.24g). A 5.20-g aliquot was separated from Fr. 1 to Fr. 7 by HPLC (Shim-pack PREP-ODS column; eluent MeOH/CH_2Cl_2 5:1v/v; flow rate 7.5ml/min).

*6-(Abieta-6',8',11',13'-tetraenyl-12'-oxy)-7-methoxyabieta-8,11,13-trien-12-ol (**1**)*

The Fr. 3 fraction (623.7mg) was separated from Fr. 31 to Fr. 33 by HPLC (Shim-pack PREP-ODS column; eluent MeOH/CH_2Cl_2 5:1 v/v; flow rate 7.5ml/min). Fr. 32 (153.6mg) was identified as 6-(abieta-6',8',11',13'tetraenyl-12'-oxy)-7-methoxyabieta-8,11,13-trien-12-ol by several spectra. Fr. 432 (44.2mg), Fr. 532 (15.1mg), Fr. 622 (7.7mg), and Fr. 722 (17.2mg) were also isolated and identified. ^{1}H-NMR ($CDCl_3$) and ^{13}C-NMR ($CDCl_3$) (Table 1) HR-MS: *m/z* 598.4395 (M^+: $C_{41}H_{58}O_3$ requires 598.4371). DI-MS: *m/z* (%) 598 (M^+; 3), 567 (2), 565 (1), 315 (12), 284 (100), 269 (17), 213 (56), 202 (93), 159 (30), 55 (49).

*Manool; 8 (17), 14-labdadien-13R-ol (**2**)*

The Fr. 2 fraction (1.08g) was separated from Fr. 21 to Fr. 25 by HPLC (Shim-pack PREP-ODS column; eluent MeOH; flow rate 7.5ml/min). Fr. 24 (304.6mg) was separated further from Fr. 241 to Fr. 243 by HPLC (Shim-pack PREP-ODS column; eluent MeOH/H_2O 9:1v/v; flow rate 7.5ml/min). Fr. 243 (200mg) was identified as manool by several spectra. ^{1}H-NMR ($CDCl_3$): δ 0.67 (s, 3H, 20-Me), 0.80 (s, 3H, 19-Me), 0.87 (s, 3H, 18-Me), 1.27 (s, 3H, 16-Me), 4.40 (s, 1H, 17-H), 4.80 (s, 1H, 17-H), 5.05 (dd, 1H, J = 10.8 and 1.3Hz, 15-H), 5.20 (dd, 1H, J = 17.4 and 1.3Hz, 15-H), 5.90 (dd, 1H, J = 17.4 and 10.8Hz, 14-H). DI-MS: *m/z* (%) 290 (M^+; 1), 272 (19), 257 (37), 137 (100), 95 (60), 81 (74).

*trans-Communic acid; 8 (17), 12, 14-labdatrien-19-oic acid (**3**)*

The Fr. 22 fraction (126.4mg) was identified as *trans*-communic acid by several spectra. ^{1}H-NMR ($CDCl_3$): δ 0.65 (s, 3H, 20-Me), 1.24 (s, 3H, 19-Me), 1.75 (s, 3H, 16-Me), 4.47 (s, 1H, 17-H), 4.84 (s, 1H, 17-H), 4.88 (d, 1H, J = 10.6Hz, 15-H), 5.04 (d, 1H, J = 17.6Hz, 15-H), 5.41 (t, 1H, J = 6.6Hz, 12-H), 6.33 (dd, 1H, J = 17.6 and 10.6Hz, 14-H). DI-MS: *m/z* (%) 302 (M^+; 44), 287 (27), 246 (15), 175 (51), 147 (54), 134 (61), 81 (100).

*Ferruginol (**4**)*

The Fr. 23 fraction (140.2 mg) was identified as ferruginol by several spectra. Fr. 242 (100 mg) was also isolated and identified. ^{1}H-NMR ($CDCl_3$): δ 0.91 (s, 3H, 20-Me), 0.93 (s, 3H, 19-Me), 1.16 (s, 3H, 18-Me), 1.20 (d, 3H, J = 7.0 Hz, *i*-Pr.), 1.23 (d, 3H, J = 7.0 Hz, *i*-Pr.), 3.11 (sept, 1H, J = 7.0 Hz, 15-H), 3.49 (s, 1H, OH), 6.63 (s, 1H, 11-H), 6.83 (s, 1H, 14-H). DI-MS: *m/z* (%) 286 (M^+; 100), 271 (93), 229 (15), 201 (32), 189 (49), 175 (54), 69 (44).

*Sugiol (**5**)*

The Fr. 1 fraction (1.40 g) was separated from Fr. 11 to Fr. 15 by HPLC (Shim-pack PREP-ODS column; eluent MeOH/H_2O 9:1 v/v; flow rate 7.5 ml/min). Fr. 15 (349.5 mg) was separated further from Fr. 151 to Fr. 153 by HPLC (Shim-pack PREP-ODS column; eluent MeOH/H_2O 8:2 v/v; flow rate 7.5 ml/min). Fr. 152 (58.9 mg) was identified as sugiol by several spectra. Fr. 143 (17.3 mg) was also isolated and identified. ^{1}H-NMR (DMSO-*d6*): δ 0.86 (s, 3H, 20-Me), 0.92 (s, 3H, 19-Me), 1.11 (d, 3H, J = 7.0 Hz, *i*-Pr.), 1.1356 (s, 3H, 18-Me), 1.1360 (d, 3H, J = 7.0 Hz, *i*-Pr.), 2.49 (s, 2H, 6-H), 3.12 (sept, 1H, J = 7.0 Hz, 15-H), 6.77 (s, 1H, 11-H), 7.64 (s, 1H, 14-H). DI-MS: *m/z* (%) 300 (M^+; 100), 285 (96), 257 (4), 217 (35), 203 (32), 161 (10), 121 (3).

*6α,12-Dihydroxyabieta-8,11,13-trien-7-one (**6**)*

The Fr. 14 fraction (140.3 mg) was separated further from Fr. 141 to Fr. 143 by HPLC (Shim-pack PREP-ODS column; eluent MeOH/H_2O 8:2 v/v; flow rate 7.5 ml/min). Fr. 142 (71 mg) was identified as 6α,12-dihydroxyabieta-8,11-13-trien-7-one by several spectra. Fr. 135 (28.7 mg) was also isolated and identified. ^{1}H-NMR ($CDCl_3$): δ 1.19 (s, 3H, 18-Me), 1.22 (s, 3H, 19-Me), 1.23 (d, 3H, J = 6.6 Hz, *i*-Pr.), 1.25 (d, 3H, J = 6.6 Hz, *i*-Pr.), 1.34 (s, 3H, 20-Me), 3.18 (sept, 1H, J = 6.6 Hz, 15-H), 4.63 (d, 1H, J = 12.8 Hz, 6-H), 6.73 (s, 1H, 11-H), 7.92 (s, 1H, 14-H). DI-MS: *m/z* (%) 316 (M^+; 59), 301 (42), 287 (100), 231 (56), 203 (55).

*6,12-Dihydroxyabieta-5,8,11,13-tetraen-7-one (**7**)*

The Fr. 153 fraction (49.5 mg) was identified as 6,12-dihydroxyabieta-5,8,11,13-tetraen-7-one by several spectra. ^{1}H-NMR ($CDCl_3$): δ 1.26 (d, 3H, J = 6.6 Hz, *i*-Pr.), 1.29 (d, 3H, J = 6.6 Hz, *i*-Pr.), 1.426 (s, 3H, 18- or 19-Me), 1.430 (s, 3H, 18- or 19-Me), 1.49 (s, 3H, 20-Me), 3.20 (sept, 1H, J = 6.6 Hz, 15-H), 6.87 (s, 1H, 11-H), 7.16 (s, 1H, 12-OH), 8.01 (s, 1H, 14-H). ^{13}C-NMR (DMSO-*d6* + $CDCl_3$, 5:1): δ 17.0 (2-CH_2), 22.1 (16-Me), 22.5 (17-Me), 26.2 (15-CH), 27.3 (18-Me), 27.9 (19-Me), 33.1 (1-CH_2), 35.1 (4-C), 35.2 (20-Me), 37.4 (3-CH_2), 38.0 (10-C in DMSO-*d6*), 110.6 (14-CH), 119.2 (8-C), 124.0 (11-CH), 133.6 (13-C), 139.2 (5-C), 143.7 (6-C), 153.9 (9-C), 159.4 (12-C), 178.6 (7-CO). DI-MS: *m/z* (%) 314 (M^+; 100), 299 (17), 285 (7), 271 (37), 244 (83), 217 (15), 203 (17).

*12-Hydroxy-6,7-secoabieta-8,11-13-trien-6,7-dial (**8**)*

The Fr. 13 fraction (313.1 mg) was separated further from Fr. 131 to Fr. 135 by HPLC (Shim-pack PREP-ODS column; eluent MeOH/H_2O 8:2 v/v; flow rate 7.5 ml/min). Fr. 132 (49.9 mg) was identified as 12-hydroxy-6,7-secoabieta-8,11,13-trien-6,7-dial by several spectra. ^{1}H-NMR ($CDCl_3$): δ 0.71 (s, 3H, 20-Me), 1.01 (s, 3H, 19-Me), 1.26 (d, 3H, J = 7.0 Hz, *i*-Pr.), 1.28 (d, 3H, J = 7.0 Hz, *i*-Pr.), 1.50 (s, 3H, 18-Me), 3.18 (sept, 1H, J = 7.0 Hz, 15-H), 6.96 (s, 1H, 11-H), 7.83 (s, 1H, 14-H), 9.84 (d, 1H, J = 3.7 Hz, 6-H), 10.46 (s, 1H, 7-H). DI-MS: *m/z* (%) 316 (M^+; 50), 301 (40), 287 (45), 231 (42), 217 (39), 203 (100), 86 (80).

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