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## New Caryophyllene Derivatives from *Betula litwinowii*

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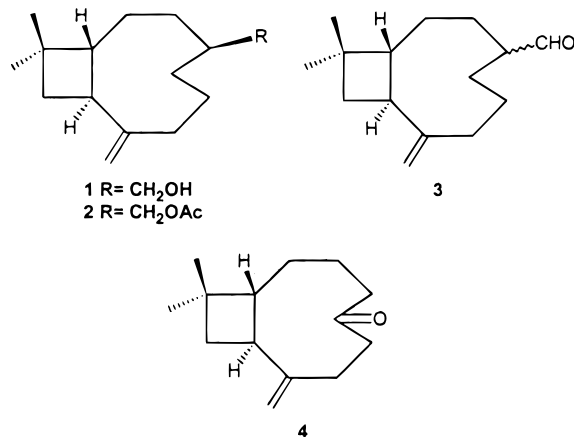
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Four new caryophyllene derivatives—14-hydroxy-4,5-dihydro- $\beta$ -caryophyllene (**1**), 14-acetoxy-4,5-dihydro- $\beta$ -caryophyllene (**2**), 4,5-dihydro- $\beta$ -caryophyllene-14-al (**3**), and caryophylla-8(14)-en-5-one (**4**)—are reported from the essential oil of *Betula litwinowii*. Compounds **1**–**4** were characterized by MS and 1D and 2D NMR analyses and chemical transformations. The antibacterial and antifungal activity of **1** is also reported.

*Betula* sp. (Betulaceae), commonly known as birch, are trees and tree-like shrubs that are widespread in temperate regions of the world. A characteristic feature of *Betula* species is their white bark. Five *Betula* species can be found in the north eastern and eastern regions of Turkey. *B. litwinowii* Doluch. is distributed in Turkey and the Caucasus at altitudes ranging from 1700 to 2800 m and has a characteristic pinkish white trunk.<sup>2,3</sup> The medicinal and cosmetic applications of *Betula* leaves, tar, and essential oils have been known since ancient times, and the essential oils obtained from *Betula* species have been the subject of many investigations.<sup>4–7</sup>

Our research on *Betula* species growing in Turkey has resulted in isolation of the new caryophyllene derivatives (**1**–**4**) from *B. litwinowii*. The biological activities of the main essential oil component, 14-hydroxy-4,5-dihydro- $\beta$ -caryophyllene (**1**), are also discussed.



### Results and Discussion

Water distillation of semi-fresh buds of *B. litwinowii* yielded a colorless essential oil with a characteristic odor. The oil was subjected to MPLC, which provided compound **1** in 25% yield from the essential oil mixture.

The HRES–FTMS of **1** provided a molecular ion [M – H]<sup>+</sup> at *m/z* 221.1906, suggesting the molecular formula C<sub>15</sub>H<sub>26</sub>O and three degrees of unsaturation. The <sup>13</sup>C NMR and DEPT spectra contained 15 carbon signals; an exocyclic double bond, two tertiary methyls, seven methylenes (one bearing oxygen), three methines, and one quaternary

carbon atom. These data, along with other spectral data, indicated the close similarity of **1** to  $\beta$ -caryophyllene.<sup>8</sup> The methyl carbons resonating at  $\delta$  22.3 and 30.3 were assigned C-12 and C-13, respectively (Table 1). These assignments were based on <sup>3</sup>*J*-HMBC correlations between H<sub>3</sub>-12 and -13 with C-1 and C-10. The geminal location of both C-12 and C-13 methyl carbons on C-11 was deduced from their <sup>3</sup>*J*-HMBC correlation to each other and their <sup>2</sup>*J*-HMBC correlation to the same quaternary carbon at  $\delta$  33.4 (C-11). The proton H-9 ( $\delta$  2.50, Table 1) displayed strong COSY couplings to H-1 ( $\delta$  1.68) and H<sub>2</sub>-10 ( $\delta$  1.66 and 1.77). H-9 also showed a <sup>3</sup>*J*-HMBC correlation with C-11, while H-1 was HMBC-correlated with C-10, which confirmed the assignments of the cyclobutane ring. The  $\Delta^{8,15}$  system was assigned on the basis of the <sup>3</sup>*J*-HMBC correlations between the exomethylene olefinic singlets at  $\delta$  4.82 and 4.88 (H<sub>2</sub>-15) and C-7 and C-9. Both H<sub>2</sub>-15 signals also displayed a <sup>2</sup>*J*-HMBC correlation to the quaternary olefinic carbon at  $\delta$  153.9 (C-8). The downfield oxygenated doublet of doublets at  $\delta$  3.37 and 3.45 (H<sub>2</sub>-14) displayed COSY coupling with H-4 ( $\delta$  1.50). H<sub>2</sub>-14 also showed <sup>3</sup>*J*-HMBC correlations with both C-3 and C-5 ( $\delta$  32.1 and 28.4, respectively). Both H<sub>2</sub>-3 signals also displayed a <sup>3</sup>*J*-HMBC correlation to C-1, confirming the assignments of the macrocycle C-1/C-9. The relative stereochemistry of **1** was resolved by comparison of <sup>13</sup>C NMR chemical shift values with literature,<sup>9</sup> molecular modeling study (Spartan 5.0), and analysis of NOESY data (Figure 1). The  $\alpha$ -oriented H-9 in **1** (Figure 1) displayed NOESY correlation with the proton multiplet at  $\delta$  2.00 (H-7a), suggesting a similar stereo orientation. The proton H-7a also displayed a NOESY correlation with H-4, which indicated the  $\beta$ -orientation of the hydroxymethylene C-14. The assignment of the relative stereochemistry of C-12 and C-13 methyl groups was primarily based on comparison of their <sup>13</sup>C NMR chemical shift values with other known caryophyllenes.<sup>9</sup> Thus, the structure of **1** was established as 14-hydroxy-4,5-dihydro- $\beta$ -caryophyllene, a new natural product.

Compound **2** displayed a molecular ion peak [M]<sup>+</sup> at *m/z* 264, suggesting the molecular formula C<sub>17</sub>H<sub>28</sub>O<sub>2</sub>. The IR spectrum of **2** displayed strong bands at 1761 and 1231 cm<sup>–1</sup>, suggesting the presence of an ester moiety. The GC retention data and <sup>1</sup>H and <sup>13</sup>C NMR data of **2** (Table 1) suggested the close similarity to **1**, with one additional acetate moiety. The proton singlet resonating at  $\delta$  2.04 correlated with the methyl carbon at  $\delta$  21.0 and was assigned to the 14-*O*-acetate group. The carbonyl carbon absorbed at  $\delta$  171.3 completed the acetate functionality. Hence, compound **2** was proved to be 14-acetoxy-4,5-

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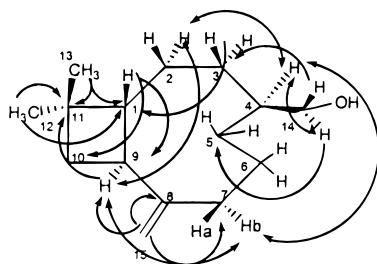
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**Table 1.**  $^{13}\text{C}$  and  $^1\text{H}$  NMR Spectral Data of Compounds **1**–**4**<sup>a</sup>

C#	<b>1</b> <sup>b</sup>		<b>2</b>		<b>3</b>		<b>4</b> <sup>b</sup>	
	$\delta_{\text{C}}$	$\delta_{\text{H}}$	$\delta_{\text{C}}$	$\delta_{\text{H}}$	$\delta_{\text{C}}$	$\delta_{\text{H}}$	$\delta_{\text{C}}$	$\delta_{\text{H}}$
1	55.9	1.68, m	55.4	1.65, 1H, m	55.7	1.65, 1H, m	54.6	1.70, m
2	30.1	1.69, 2H, m	28.1	1.73, 2H, m	26.9	1.70, 2H, m	28.0	1.72, m
3	32.1	1.07, m (Ha); 1.78, m (Hb)	29.4	1.12, m	26.3	1.19, m	24.8	1.76, m (Ha); 2.02, m (Hb)
4	40.9	1.50, m	37.1	2.43, m	43.9	2.20, m	43.7	2.40, m
5	28.4	1.34, 2H, m	31.7	1.26, m	27.4	1.25, m	215.2	
6	27.6	1.59, 2H, m	27.1	1.43, 2H, m	25.1	1.57, 2H, m	41.5	2.36, m; 2.57, m
7	35.5	2.00, m (Ha); 2.36, m (Hb)	35.1	2.30, m	29.6	2.20, m	34.9	1.80, m (Ha); 2.36, m (Hb)
8	153.9		153.4		153.8		152.7	
9	46.3	2.50, dd (8.8, 9.3)	45.8	2.45, m	49.1	2.35, m	45.7	2.29, dd (9.4, 9.8)
10	37.7	1.66, m (Ha); 1.77, m (Hb)	37.4	1.63, 2H, m	38.6	1.61, 2H, m	39.1	1.68, m
11	33.4		33.5		33.5		33.5	
12	22.3	1.00, 3H, s	21.9	0.98, 3H, s	21.6	0.95, 3H, s	22.3	1.01, 3H, s
13	30.3	1.00, 3H, s	29.9	0.98, 3H, s	29.8	0.91, 3H, s	30.3	1.04, 3H, s
14	68.7	3.37, dd (4.3, 10.4) 3.45, dd (6.1, 10.4)	69.4	3.86, 2H, d (6.4)	205.2	9.47, s	111.6	4.91, s; 4.99, d (1.9)
15	109.8	4.82, s; 4.88, s	109.6	4.84, 2H, d (4.3)	109.7	4.80, 2H, m		
14-OAc			21.0	2.04, 3H, s				
14-OAc			171.3					

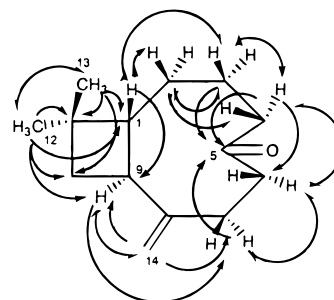
<sup>a</sup> In  $\text{CDCl}_3$ , 22.4 MHz for  $^{13}\text{C}$  NMR and 90 MHz for  $^1\text{H}$  NMR. Coupling constants ( $J$ ) are in Hz. <sup>b</sup> At 125 MHz for  $^{13}\text{C}$  NMR and 500 MHz for  $^1\text{H}$  NMR.

**Figure 1.** Important HMBC (plain arrow) and NOESY (two-sided arrow) correlations for compound **1**.

dihydro- $\beta$ -caryophyllene, which was also present naturally in the essential oil compared by GC–MS.

Compound **3** was purified by MPLC of the essential oil. The molecular formula ( $\text{C}_{15}\text{H}_{24}\text{O}$ ) was deduced from GC–MS and  $^{13}\text{C}$  NMR. The IR spectrum indicated a saturated aldehyde with the absorptions at 2807, 2697, and 1739  $\text{cm}^{-1}$ . Compound **3** displayed spectral data closely similar to those of **1** (Table 1). The  $^1\text{H}$  NMR spectrum of **3** contained a signal characteristic for an aldehyde at  $\delta$  9.48, which correlated with the downfield methine carbon resonating at  $\delta$  205.2 (C-14). Kaiser and Lamparsky<sup>11</sup> reported the occurrence of the related caryophyllen-14-al (=  $\alpha$ -betulenal) in lavender oil and its synthesis. The instability of **3** hindered any attempts to determine the stereochemistry of C-4. Hence, compound **3** was assigned as 4,5-dihydro- $\beta$ -caryophyllen-14-al.

Compound **4** was isolated from the oil using the same procedure as explained above. The GC–FTIR spectrum indicated a characteristic ketone band at 1720  $\text{cm}^{-1}$ . The HRES–FTMS showed a molecular ion  $[\text{M} + \text{H}]^+$  at  $m/z$  207.1740 ( $\text{C}_{14}\text{H}_{22}\text{O}$ ). The MS data supported the proposed formula, as did the NMR data, which were similar to those of **1**. The downfield ketone carbon resonating at  $\delta$  215.2 was assigned to C-5 (Table 1). This was based on its  $^3J$ -HMBC correlations with  $\text{H}_2$ -3 ( $\delta$  2.02 and 1.76) and  $\text{H}_2$ -7 ( $\delta$  2.36 and 1.80). Also H-4 ( $\delta$  2.40) and H-6a ( $\delta$  2.36) correlated with C-5, along with supporting COSY correlations in two different spin systems (see Figure 2). Hence, compound **4** was assigned as the new natural product caryophylla-8(14)-en-5-one (**4**). This compound was previously reported as a synthetic product from  $\beta$ -caryophyllene along with other derivatives by Matsubara et al.<sup>12</sup> However, the provided data and stereochemistry were tentative.

**Figure 2.** Important  $^1\text{H}$ – $^1\text{H}$  COSY (two sided arrow) and HMBC (plain arrow) correlations for compound **4**.**Table 2.** Antimicrobial Activity of Compound **1**<sup>a</sup>

microorganism	standard MIC $\mu\text{g/mL}$	<b>1</b> MIC $\mu\text{g/mL}$
<i>E. coli</i>	250 <sup>b</sup>	250
<i>S. aureus</i>	7.81 <sup>b</sup>	250
<i>M. luteus</i>	125 <sup>b</sup>	250
<i>P. aeruginosa</i>	62.5 <sup>b</sup>	250
<i>B. cereus</i>	125 <sup>b</sup>	125
<i>C. glabrata</i>	62.5 <sup>c</sup>	125

<sup>a</sup> Using broth susceptibility assay.<sup>14</sup> <sup>b</sup> Chloramphenicol. <sup>c</sup> Ketoconazole.

Compound **1** induced 100% inhibition of the plant pathogenic fungi *Cephalosporium aphidicola* and *Rhizoctonia cerealis* at 200  $\mu\text{g/mL}$ . Compound **1** was less effective against *Drechslera sorokiniana* and *Fusarium solani* by displaying 75 and 59% inhibition, respectively, as compared to the 100% inhibition induced by the standard antifungal ketoconazole.

Antimicrobial activity of **1** against several human pathogens is presented in Table 2. Compound **1** was as active as the antibacterial standard chloramphenicol against *Bacillus cereus*, with a MIC value of 125  $\mu\text{g/mL}$ , but was less active against *Escherichia coli*, *Micrococcus luteus*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*. Compound **1** displayed moderate antifungal activity against *Candida glabrata*, with a MIC value of 125  $\mu\text{g/mL}$ , compared with ketoconazole (62.5  $\mu\text{g/mL}$ ).

## Experimental Section

**General Experimental Procedures.** TLC plates were prepared using Si gel 60G (Merck 7731) + Si gel 60GF<sub>254</sub> (Merck 7730) in equal proportions. Compounds were visualized with UV light (365 and 254 nm) and developed with 1%

vanillin–5% H<sub>2</sub>SO<sub>4</sub> in EtOH followed by heating. Separations were performed using a Büchi B-680 MPLC system. Si gel 60G (Merck 7734) was used as packing material, filled wet with hexane (column size: 26 × 460 mm). Hexane–diethyl ether was gradient eluted with a flow rate of 10 mL/min, at 14 bar pressure. Optical rotations were measured on an Oriel Pol S-2. IR were measured using a GC–FTIR Perkin-Elmer Spectrum 2000. <sup>1</sup>H and <sup>13</sup>C NMR spectra were recorded on a JEOL JNM-EX90A at 90 and 22.4 MHz, respectively, in CDCl<sub>3</sub>. Tetramethylsilane (TMS) at 0.0 ppm was referenced as an internal standard. Additionally, 1D and 2D NMR data were obtained using Bruker Avance DPX-400 and 500 spectrometers. Spectra were measured and reported in parts per million (ppm) by using the residual solvent peak as an internal standard. MS were recorded using a HP G1800A GCD system. Innosil FSC column (60 m × 0.25 mm i.d., with 0.25 μm film thickness) was used with helium (1 mL/min) as carrier gas. GC oven temperature was kept at 60 °C for 10 min and programmed to 220 °C at a rate of 4 °C/min, then kept constant at 220 °C for 10 min to 240 °C at rate of 1 °C/min. Mass range was recorded from *m/z* 35 to 425. MS were measured at 70 eV. Relative percentages of the individual compounds were calculated automatically from peak areas of the total ion chromatogram. ES–FTMS analyses were measured on a Bruker-Magnex BioAPEX 30es ion cyclotron HRHPLC–FTMS spectrometer by direct injection into an electrospray interface.

**Plant Material.** *B. litwinowii* was collected in May 1998, from Artvin, Hatila, 2050 m. Voucher specimens are kept at the Faculty of Pharmacy Herbarium at Anadolu University in Eskişehir, Turkey (ESSE 12757).

**Distillation and Isolation.** Air-dried buds of *B. litwinowii* were water distilled for 4 h using a Clevenger-type apparatus to yield 6.34% essential oil on a dry-weight basis. *B. litwinowii* bud essential oils were used for MPLC separations yielding the following compounds.

**14-Hydroxy-4,5-dihydro-β-caryophyllene (1):** essential oil (0.6 g) subjected to MPLC over Si gel, eluted with hexane–diethyl ether (7:3) to yield **1** (150 mg); [α]<sub>D</sub><sup>25</sup> –11.6° (*c* 2.1, EtOH), GC–FTIR *ν*<sub>max</sub> (cm<sup>–1</sup>) 3669 (OH) 3082, 1634 and 890 (C=CH<sub>2</sub>), 1374 [C(CH<sub>3</sub>)<sub>2</sub>]; <sup>1</sup>H NMR and <sup>13</sup>C NMR (δ, ppm), see Table 1; EIMS *m/z* 222 [M]<sup>+</sup> (2), 207 (7), 191 (23), 147 (10), 135 (35), 121 (31), 109 (48), 95 (100), 79 (74), 67 (61), 55 (44), 41 (76); HRES–FTMS *m/z* 221.1906 [M – H]<sup>+</sup> (calcd for C<sub>15</sub>H<sub>26</sub>O: 221.1905).

**14-Acetoxy-4,5-dihydro-β-caryophyllene (2):** acetylation of **1** (26 mg) in pyridine (1 mL) with acetic anhydride (1 mL) yielded **2** (22 mg); [α]<sub>D</sub><sup>25</sup> –10.2° (*c* 2.0, EtOH); GC–FTIR *ν*<sub>max</sub> (cm<sup>–1</sup>) 3082, 1633 and 891 (C=CH<sub>2</sub>), 1761 (C=O), 1231 and 1035 (OAc); <sup>1</sup>H NMR and <sup>13</sup>C NMR (δ, ppm), see Table 1; EIMS *m/z* 264 [M]<sup>+</sup> (1.0), 249 (1), 204 (9), 176 (13), 161 (22), 147 (16), 133 (40), 119 (32), 108 (54), 93 (55), 82 (79), 79 (53), 67 (41), 55 (27), 43 (100).

**4,5-Dihydro-β-caryophyllen-14-al (3):** essential oil (0.6 g) subjected to MPLC over Si gel, eluted with hexane–diethyl ether (96:4) to yield **3** (10 mg); [α]<sub>D</sub><sup>25</sup> –6.0° (*c* 2.0, EtOH); GC–FTIR *ν*<sub>max</sub> (cm<sup>–1</sup>) 3079, 1634 and 899 (C=CH<sub>2</sub>), 2807, 2697 and 1739 (CHO), 2937; <sup>1</sup>H NMR and <sup>13</sup>C NMR (δ, ppm) see Table 1; EIMS *m/z* 220 [M]<sup>+</sup> (2.0), 205 (4), 177 (5), 135 (15), 121 (18), 107 (26), 95 (33), 82 (100), 79 (43), 67 (43), 55 (25), 41 (53).

**Caryophylla-8(14)-en-5-one (4):** essential oil (0.6 g) subjected to MPLC over Si gel, eluted with hexane–diethyl ether (95:5) to yield an oil, **4** (19 mg); [α]<sub>D</sub><sup>25</sup> –14.5° (*c* 1.0, EtOH); UV (CHCl<sub>3</sub>) λ<sub>max</sub> (log *e*) 243 (3.11); GC–FTIR *ν*<sub>max</sub> (cm<sup>–1</sup>) 3079, 1634 and 892 (C=CH<sub>2</sub>), 1720 (C=O), 2956, 2874, 1452; <sup>1</sup>H NMR and <sup>13</sup>C NMR (δ, ppm), see Table 1; EIMS *m/z* 206 [M]<sup>+</sup> (2.0), 191 (15), 189 (15), 163 (15), 145 (38), 124 (64), 82 (92), 79 (88), 55 (97), 41 (100); HRES–FTMS *m/z* 207.1740 [M + H]<sup>+</sup> (calcd C<sub>15</sub>H<sub>26</sub>O: 207.1749).

**Antifungal Bioassay.** Antifungal activity was determined using an agar tube dilution technique. Stock solution of **1** was freshly prepared in DMSO to reach a final concentration of 200 μg/mL using sterile molten SDA. Test tubes were kept at room temperature for solidification. Medium containing only

DMSO was used as negative control. Fungal cultures were cut to 4 × 4 mm from 1-week grown cultures and then inoculated onto the slant. After an incubation period of 7–10 days at 29 °C, tubes were examined for growth inhibition. Ketoconazole was used as a reference antifungal drug. Media growth with the compound was determined by measuring linear growth (mm) of fungal culture. Growth inhibition (%) was calculated with reference to the negative control.<sup>13</sup> The plant pathogenic fungi was obtained from the culture collection of the Biology Department at the Faculty of Science and Letters, Anadolu University.

**Antimicrobial Bioassay.** A stock solution of pure compound was prepared in DMSO and serially diluted from 1000 μg/mL to 0.97 μg/mL in sterile distilled water. Freshly grown bacterial suspensions in double strength Mueller-Hinton broth and yeast suspension of *C. glabrata* in yeast medium were standardized to 108 CFU/mL. Each dilution of the antimicrobial agent (100 μL) was transferred to a 96-well microtiter plate. Sterile distilled water served as growth control. Each microbial suspension (100 μL) was then added to each well. The last row, containing only the serial dilutions of antimicrobial agent without microorganism, was used as negative control. After incubation at 37 °C for 24 h the first well without turbidity was determined as the minimal inhibition concentration (MIC).<sup>14</sup> Human pathogens used for this assay were obtained from the culture collection of Osmangazi University, Medical Faculty, Microbiology Department; *E. coli*, *S. aureus*, *M. luteus*, *P. aeruginosa*, *B. cereus* and the fungus *C. glabrata* (Table 2).

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