Antioxidant and Anticholinesterase Activity Evaluation of *ent*-Kaurane Diterpenoids from *Sideritis arguta*[⊥]

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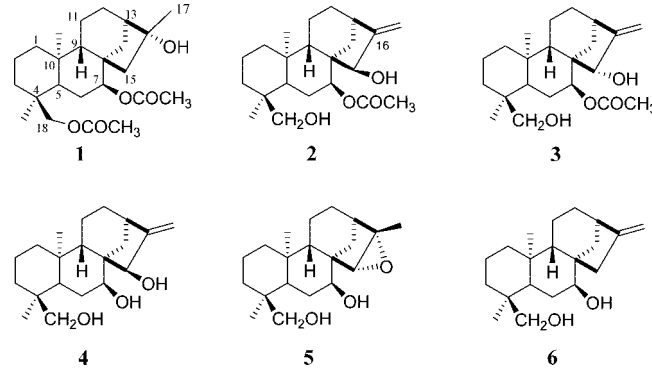
The petroleum ether and acetone extracts of the aerial parts of *Sideritis arguta* afforded two new and six known diterpenoids with the *ent*-kaurane skeleton. The structures of the new diterpenoids were determined as *ent*-7α,18-diacetoxy-16*β*-hydroxykaurane (diacetyldistanol) (**1**) and *ent*-7α-acetoxy,15α,18-dihydroxykaur-16-ene (15-*epi*-eubol) (**2**) by spectroscopic data interpretation. Antioxidant potential was investigated for the *ent*-kauranes and the plant extracts by three methods (*β*-carotene bleaching, free-radical scavenging, and superoxide-anion scavenging activity). Acetylcholinesterase and butyrylcholinesterase inhibitory activity were also evaluated, and the *ent*-kauranes eubol (**3**), sideroxol (**5**), and 7-*epi*-candicandiol (**6**) exhibited moderate butyrylcholinesterase inhibitory activity.

The genus *Sideritis* (Lamiaceae) is represented by about 150 species altogether worldwide, which occur mainly in temperate and tropical regions of the Northern Hemisphere, particularly in the Mediterranean and the Middle East.[1] In Turkey, this genus is represented by 55 taxa.[2−4] Previous studies on the isolation of diterpenoids and phenolics from *Sideritis* species have been performed mainly by researchers in the Canary Islands, elsewhere in Spain, and in Italy, and the reported diterpenoids have different carbon skeleta[5−7] inclusive of *ent*-kauranes.[8,9]

Sideritis plant extracts and their constituents have been found to have anti-inflammatory, antirheumatic, antiulcer, insecticidal, antifeedant, antimicrobial, antioxidant, and cytotoxic activities.[5,10−15] *Sideritis* species are traditionally used to fight the common cold, flu, and allergies as well as being gastroprotective and digestive agents, and they are prepared as folk medicines from their dried inflorescenses.[15] In Anatolia, *Sideritis* species are known as "mountain tea" or "plateau tea".

Sideritis arguta Boiss. and Heldr. is widespread throughout Turkey, and its local name is "eşek çayı", "donkey tea". The essential oil of *S. arguta* is used for flavoring foods, in the pharmaceutical, cosmetic, and perfumery industries, and in folk medicine. In a report[16] on antioxidant activity evaluation of selected Anatolian *Sideritis* species, *S. arguta* was one of the species studied. However, only its water extract was investigated by a thiobarbutiric acid system, and no phytochemical study has been performed previously. Interest has increased in naturally occurring antioxidants since they may be used to protect humans from oxidative stress damage.[17] The use of antioxidants may slow the progression of Alzheimer's disease (AD) and minimize neuronal degeneration.[18,19] Thus, in continuation of our chemical investigations on plants of the Lamiaceae,[10] the antioxidant and anticholinesterase activities of *S. arguta* were investigated. Two new *ent*-kaurane diterpenoids (**1** and **2**) were isolated, and their structures were elucidated, particularly on the basis of 1D- and 2D-NMR experiments.

The IR spectrum of compound **1** exhibited a hydroxyl absorption at 3429 cm^{-1} and acetyl absorptions at 1722 (−C═O) and 1245 cm^{-1} (−O−C═). In the ^1H NMR spectrum ($CDCl_3$) (Table 1), three



methyl signals were observed as singlets at *δ* 0.74, 1.01, and 1.30. The last signal was indicative of a methyl group adjacent to an oxygen moiety, as either a hydroxyl or an epoxy. Two acetate methyl proton signals were observed at *δ* 1.95 and 1.97. One of the acetoxy groups could be located on a secondary carbon, since its methine proton resonated at *δ* 4.75, while the other acetyl group was observed as an acetoxymethylene group with an AB pair of methylene signals at *δ* 3.57 and 3.72, having a coupling value of 11.5 Hz. The ^{13}C NMR (APT experiment) (Table 1), displaying 24 C atoms, verified the presence of two acetyl moieties on a diterpene structure consisting of five methyls, nine methylenes, four methines, and six quaternary carbon atoms. Three methyl carbon signals belonging to an *ent*-kaurane skeleton were observed at *δ* 17.5, 18.2, and 24.6, corresponding to C-20, C-19, and C-16, respectively. An oxygenated methine carbon was observed at *δ* 80.1 (C-7), and an oxygenated methylene carbon resonated at *δ* 72.8 (C-18). The third oxygenated carbon signal appeared at *δ* 79.1 and was assigned as a quaternary carbon atom (C-16), and its placement was established by a HMBC experiment. A downfield shift of the C-13 signal to 48.9 ppm verified the position of this tertiary hydroxyl group at C-16. The acetate carbonyl signals were observed at *δ* 171.1 and 172.4, while their methyl carbons resonated at *δ* 21.3 and 21.5.

The HREIMS of **1** exhibited a molecular ion peak at *m*/*z* 406.2738, corresponding to the molecular formula $C_{24}H_{38}O_5$. In the (+) APCIMS, the molecular peak was observed at *m*/*z* 406, and subsequent losses of two acetoxy groups were observed at *m*/*z* 347 and 287, and a base peak at *m*/*z* 269. Therefore, the structure of compound **1** was established as *ent*-7α,18-diacetoxy-16*β*-hydroxykaurane (**1**). Compound **1** has been previously prepared as a

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Table 1. NMR Spectroscopic Data (500 MHz, $CDCl_3$) for *ent*-Kauranes **1** and **2**[a]

	diacetyldistanol (**1**)		15-*epi*-eubol (**2**)	
position	δ_C	δ_H	δ_C	δ_H
1	39.7		42.0	1.78 dt (2.0, 10.4) 0.81 brt (10.3)
2	17.9		22.2	1.44 m
3	35.9		35.3	1.72 m 1.46 td (2.0, 11.0)
4	36.4		37.2	
5	42.0		42.0	1.77 brd (1.2)
6	25.0		25.1	1.54 m 1.59 m
7	80.1	4.75 t (3.0)	70.2	5.02 t (1.2)
8	48.0		47.2	
9	52.1		46.3	1.45 d (8.2)
10	39.2		41.1	
11	17.6		18.3	1.55 m (2H)
12	27.6		32.2	1.45 m (2H)
13	48.9		41.2	2.78 m
14	36.4		35.3	1.74 m 1.60 m
15	54.4		84.2	4.23 s
16	79.1		156.0	
17	24.6	1.30 s	109.8	5.12 s 5.29 s
18a	72.8	3.72 (11.5)	71.3	3.26 d (10.7)
18b		3.57 (11.5)		2.96 d (10.7)
19	18.2	0.74 s	18.8	0.67 s
20	17.5	1.01 s	18.2	1.02 s
O-CO-$\underline{CH_3}$	21.3, 21.5	1.95 s, 1.97 s	21.8	2.00 s
O-$\underline{CO}$-CH_3	170.4, 170.9		170.5	

[a] Carbons were determined on the basis of APT, HSQC, and HMBC experiments.

derivative of distanol;[20] however, this is its first isolation from a natural source.

Compound **2** exhibited a molecular ion peak at *m/z* 362.2497 (calcd for $C_{22}H_{34}O_4$, 362.2457) in the HREIMS. The ^1H and ^{13}C NMR spectroscopic data (Table 1) were similar to those of the known *ent*-kauranes eubol (**3**) and eubotriol (**4**), also obtained in this study. Eubol and eubotriol were first isolated from *S. euboea* by Venturella and Bellino.[21] The main difference between eubol and compound **2** was observed for the H-15 and H-17 protons, and small chemical shift differences occurred for H-7 and H-13 (Table 1), while the ring A protons resonated at the same frequencies. Both eubol (**3**) and eubotriol (**4**) have the same stereochemistry at C-7 and C-15, and their ^1H NMR data obtained in the present study were in a good agreement with those in the literature.[21] Acetylation of both eubol (**3**) and eubotriol (**4**) afforded triacetyleubotriol, which gave very comparable chemical shift values with the semisynthetically obtained eubotriol triacetate.[21] However, the H-15 signal of compound **2** resonated at δ 4.23 as a singlet, while it resonated at δ 3.96 as a broad singlet in eubol (**3**) with β-configuration. In compound **2**, the stereochemistry at C-15 was deduced by a NOESY experiment, which showed a NOESY correlation between H-7 and H-15, indicating their α-orientations. Its (+) APCIMS gave a molecular ion peak at *m/z* 363, corresponding to $[M + 1]^+$, and fragment ions at *m/z* 344 $[M - H_2O]^+$ and 321 $[M - Ac]^+$ and a base peak at *m/z* 307. The loss of an acetoxyl group was followed by a fragment ion at *m/z* 303. Thus, compound **2** was determined to be *ent*-7α-acetoxy-15α,18-dihydroxykaur-16-ene (15-*epi*-eubol), an epimer of eubol.

The three plant extracts were investigated for antioxidant activity in the three test systems consisting of a β-carotene bleaching method and measuring free-radical scavenging activity and superoxide-anion scavenging activity. The methanol and acetone extracts showed similar activity results for the three test systems (Table 2). Among the tested *ent*-kauranes, only 7-*epi*-candicandiol (**6**) showed lipid peroxidation inhibitory activity (Table 2). All antioxidant activity tests were carried out at four concentrations, and the results are presented as IC_{50} values in Table 2.

Table 2. Antioxidant Activity Results[a,b]

sample	β-carotene−linoleic acid assay IC_{50} (μg/mL)	DPPH assay IC_{50} (μg/mL)	$O_2^{\bullet-}$ assay IC_{50} (μg/mL)
pet. ether extract	54.2 ± 1.1	NA[d]	158.94 ± 3.2
acetone extract	4.79 ± 0.7	23.61 ± 0.4	92.60 ± 1.0
methanol extract	2.6 ± 0.6	35.54 ± 0.4	124.48 ± 2.0
6	43.1 ± 0.5	NA[d]	NA[d]
α-toc[c]	2.1 ± 0.1	12.26 ± 0.1	23.25 ± 1.4
BHT[c]	1.3 ± 0.0	66.73 ± 1.1	NT[e]
BHA[c]	1.5 ± 0.0	5.88 ± 0.2	27.10 ± 0.9

[a] IC_{50} values represent the means ± standard deviation of three parallel measurements ($p < 0.05$). [b] Tested diterpenoids exhibiting IC_{50} values > 200 μM are not given in the table. [c] Reference compounds. [d] NA: not active. [e] NT: not tested.

Table 3. Anticholinesterase Activity Results[a,b]

sample	AChE IC_{50} (μM)	BChE IC_{50} (μM)
1	NA[d]	175.8 ± 2.0
3	NA[d]	23.2 ± 3.2
4	NA[d]	98.1 ± 2.6
5	14.5 ± 0.8	25.0 ± 3.0
6	22.8 ± 0.7	21.1 ± 5.1
galanthamine[e]	73.9 ± 0.8	50.9 ± 0.9

[a] IC_{50} values represent the means ± standard deviation of three parallel measurements ($p < 0.05$). [b] Tested diterpenoids and the three extracts exhibiting IC_{50} values > 200 μM are not given in the table. [c] In μg/mL concentration. [d] NA: not active. [e] Standard drug.

Neither the extracts nor diterpenes isolated showed any inhibitory activity against the acetylcholinesterase enzyme, but against butyrylcholinesterase, *ent*-kauranes eubol (**3**), sideroxol (**5**), and 7-*epi*-candicandiol (**6**) showed moderate activity (Table 3).

Experimental Section

General Experimental Procedures. Melting points were obtained on a Reichert-Kofler. Optical rotations were obtained with an AA-5 polarimeter. The spectra were recorded with the following instruments: IR spectra on a Perkin-Elmer 1615; NMR spectra on a Varian-Innova-500 Defne, 500 and 125 MHz for ^1H and ^{13}C NMR, respectively, and on a Varian Mercury-Vx 400, 400 and 100 MHz for ^1H and ^{13}C NMR, respectively; mass spectra on a (+)AP-CIMS Thermo-LCQ Deca XP-MAX; HREIMS on JEOL HX 110 mass spectrometers.

Silica gel 60 was used for column chromatography, and Kieselgel $60F_{254}$ (E. Merck), for preparative TLC as precoated plates. For antioxidant activity tests, β-carotene, linoleic acid, polyoxyethylene sorbitan monopalmitate (Tween-40), 1,1-diphenyl-2-picrylhydrazyl (DPPH), butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), α-tocopherol (α-toc), and nicotinamide adenine dinucleotide (NADH) were obtained from E. Merck (Darmstadt, Germany). For anticholinergic activity tests acetylcholinesterase (AChE), butyrylcholinesterase (BChE), 5,5′-dithiobis (2-nitrobenzoic) acid (DTNB), galanthamine, acetylthiocholine iodide, and butyrylthiocholine chloride were obtained from Sigma Chemical Co. (Sigma-Aldrich GmbH, Sternheim, Germany). Nitrotetrazolium blue chloride (NBT) and *N*-methylphenazoniummethyl sulfate (PMS) were obtained from Fluka Chemie (Fluka Chemie GmbH, Sternheim, Germany). Other chemicals used were of analytical grade.

Plant Material. The aerial parts of *Sideritis arguta* Boiss. & Heldr. were collected in Antalya City, Manavgat Province, Turkey, in July 2003. The plant was identified by Dr. Tuncay Dirmenci, Department of Secondary Science and Mathematics Education, Faculty of Education, Balıkesir University, Balıkesir, Turkey. A voucher specimen was deposited as Herbarium Number 2295, Dr. Dirmency's special collection, at the Department of Biology, Balıkesir University, Turkey.

Extraction and Isolation. The powdered plant material (1.5 kg) was extracted successively with petroleum ether and acetone to give 26.5 and 34.36 g of each extract on drying, respectively. Each extract was fractionated on a silica gel column. The petroleum ether extract was first eluted with petroleum ether and then in gradients with

dichloromethane, acetone, and methanol, respectively. From the petroleum ether extract, five diterpenoids, epoxysiderol (5.2 mg), siderol (80.0 mg), sideroxol (**5**) (100.0 mg), 15-*epi*-eubol (**2**) (5.0 mg), and eubol (**3**) (12.2 mg), were obtained successively during elution with dichloromethane−acetone (95:05−90:10). The latter two compounds were separated from each other by repeated preparative TLC purifications using a dichloromethane−acetone (90:10) solvent system. The acetone extract was also eluted with the same solvents as for the petroleum ether extract, but elution ended with 100% methanol. Four *ent*-kauranes, diacetyldistanol (**1**) (6.0 mg), epoxysiderol (3.3 mg), 7-*epi*-candicandiol (**6**) (2.0 g), and eubotriol (**4**) (3.6 mg), were obtained, successively, with elution of petroleum ether−dichloromethane (05:95) and dichloromethane−acetone (85:15). The *ent*-kaurane diacetyldistanol (**1**) was purified using dichloromethane−acetone (95:05) on preparative TLC plates to afford 6 mg of pure compound.

Diacetyldistanol (1): amorphous solid; mp 210−213 °C; $[\alpha]^{24}_{D}$ −49 (*c* 0.05, $CHCl_3$); IR ($CHCl_3$) ν_{max} 3429, 1722, 1245, 1640, 900 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) (Table 1); ^{13}C NMR ($CDCl_3$, 125 MHz) (Table 1); (+) APCIMS *m/z* 406 $[M]^+$ (2), 347 (4), 329 (78), 315 (9), 287 (28), 269 (100); HREIMS *m/z* 406.2738 (calcd for $C_{24}H_{38}O_5$, 406.2719).

15-*epi*-Eubol (2): amorphous solid; mp 186−188 °C; $[\alpha]^{24}_{D}$ −12 (*c* 0.08, $CHCl_3$); IR ($CHCl_3$) ν_{max} 3450−3320, 1724,1635,1262, 890 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) (Table 1); ^{13}C NMR ($CDCl_3$, 125 MHz) (Table 1); (+) APCIMS *m/z* 363 $[M + 1]^+$ (18), 344 (13), 321 (82), 335 (60), 307 (100), 305 (71), 303 (55), 289 (57), 285 (56), 279 (46), 271 (38), 261 (35), 255 (24), 242 (21), 227 (17), 199 (17); HREIMS *m/z* 362.2497 (calcd for $C_{22}H_{34}O_4$, 362.2457).

Acetylation of Eubol (3) and Eubotriol (4). Acetylation of each *ent*-kaurane individually afforded triacetyleubotriol. Compounds (each 3 mg) were treated with Ac_2O (2 mL)−pyridine (1 mL) at room temperature overnight to afford the same triacetyl derivative.

Triacetyleubotriol: amorphous solid; ^1H NMR ($CDCI_3$, 400 MHz) δ 0.82 (3H, s, Me-19), 1.10 (3H, s, Me-20) 1.96, 1.98, 2.04 (3H, each, OAc), 2.84 (1H, m, H-13), 3.60 (1H, d, J = 11.8 Hz, H-18b), 3.77 (1H, d, J = 11.8 Hz, H-18a), 4.96 (1H, dd, J = 2 and 4 Hz, H-7α), 5.04 (1H, brs, H-17a), 5.21 (1H, s, H-17b), 5.41 (1H, brs, H-15).

Determination of Antioxidant Activity by a β-Carotene Bleaching Method. The antioxidant activity of the extracts and the diterpenoids was evaluated by a β-carotene−linoleic acid model system, as previously described.[22]

Free-Radical Scavenging Activity. The free-radical scavenging activity of the extracts and the diterpenoids was determined by the DPPH assay, as previously described by Blois.[23]

Superoxide-Anion Scavenging Activity. Measurement of superoxide anion scavenging activity of the extracts and the diterpenoids was determined by the NADH-PMS-NBT method, as described by Liu et al.[24]

Anticholinesterase Activities. Acetylcholinesterase- and butyrylcholinesterase-inhibiting activities were measured by the spectrophotometric method developed by Ellman et al.[25]

Statistical Analysis. All data on all antioxidant activity tests are the average of triplicate analyses. The data were recorded as means ± standard deviations. Analysis of variance was performed by ANOVA procedures. Significant differences between means were determined by the Student *t* test, with p values of <0.05 being regarded as significant.

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Supporting Information Available: ^1H and ^{13}C NMR spectra for compounds **1** and **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

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