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SESQUITERPENES FROM NONCAPITATE GLANDULAR TRICHOMES OF *HELIANTHUS ANNUUS*

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Key Word Index—*Helianthus annuus*; Asteraceae; sunflower; sesquiterpenes; glandular trichomes; biological activity.

Abstract—Leaf extracts of the common sunflower *Helianthus annuus*, in addition to known sesquiterpene lactones, afforded three new sesquiterpenes, the structures of which were elucidated by spectroscopic analysis. The localization of the compounds in noncapitate glandular hairs was demonstrated by trichome microsampling in combination with HPLC analysis. All three compounds possess antimicrobial activity.

INTRODUCTION

The chemical constitution of the aerial parts of the common sunflower, *Helianthus annuus* L. has been investigated previously [1–4]. In addition to various sesquiterpene lactones, the dominant secondary metabolites of the genus [5–7], flavonoid aglycones [8], diterpenes [9, 10], coumarins [11, 12] and other derivatives of the phenylpropanoid pathway [13] have been reported. Microscopic investigation revealed the existence of two types of glandular trichomes on the leaf surface [14]. While previous studies had shown that capitate glandular trichomes are the source of sesquiterpene lactones (STL) in *Helianthus* and related genera [15], no information on the chemical constitution of the noncapitate glandular hairs was available. In continuation of our investigation of the secondary metabolites of the cultivated sunflower *H. annuus*, we now report on the content of this latter type of leaf gland.

RESULTS AND DISCUSSION

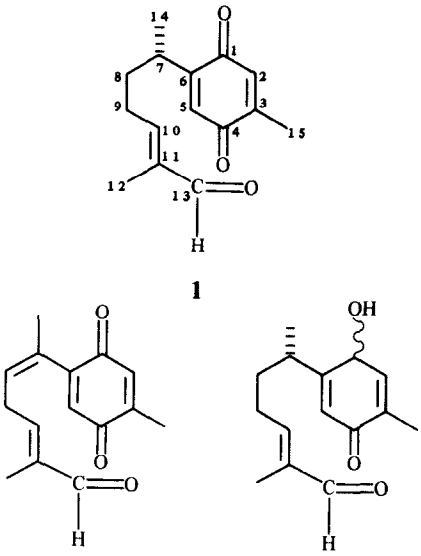
Both types of glandular trichomes are distinctive. The STL-containing hairs usually occur in small depressions of the intercostal area of leaves, most frequently on the lower surface of sunflower leaves. They consist of a multicellular basis and the terpenoids are sequestered by the tip cells into a capitate-like subcuticular cavity. In contrast, the second type of glandular trichome consists of a linear row of approximately six to 10 cells. Two or three cells at the tip are usually filled with a brownish yellow material (Fig. 1). These latter hairs are located along the leaf veins and the petiole, and range in length from about 30 to 80 μm.

Chromatographic separation of various extracts (MeOH, CH_2Cl_2, $CHCl_3$) of mechanically-harvested noncapitate glandular trichomes on TLC (silica gel 60; solvent CH_2Cl_2–Me_2CO–EtOAc, 5:4:1) showed similar patterns with two migrating zones at R_f 0.65 and 0.30. HPLC analysis (RP 18; solvent MeOH–H_2O, 3:2) of the trichome extracts revealed the presence of two major and at least one minor compound. The latter separation was used as a guide for the purification of the constituents from crude leaf extracts on a preparative scale.

Compound **1** was purified from an HPLC fraction with a retention time (R_t) of 1.72 relative to the internal standard dimethyl phenol (DMP; $R_t = 1.00$). Spectroscopic data indicated similarity with bisabolen-like sesquiterpenes [16–19]. The molecular ion in the mass spectrum at m/z 246 was in accord with $C_{15}H_{18}O_3$. Strong IR absorption at 1673 and 1651 cm^{-1} indicated the presence of α,β-unsaturated aldehyde and carbonyl functions, respectively. The structure of compound **1** was deduced from ^1H NMR (Table 1) and ^{13}C NMR spectra (Table 2) by means of COSY, ^1H-decoupling and ^{13}C-APT experiments. A proton signal at δ9.38 and a ternary carbon signal at δ195.1 confirmed the aldehyde function at C-13. ^1H NMR decoupling experiments established the sequence of H-10 (δ6.45) to H-7 (δ2.97). Irradiation at δ2.97 collapsed a three-proton signal at δ1.17, thus indicating the attachment of a methyl group at C-7. At the same time a small coupling ($J = 1$ Hz) with the second of the three olefinic protons became visible at δ6.54 (H-5). The third olefinic signal at δ6.61 (H-2) showed coupling only with the methyl group at δ2.05, thus indicating the isolation of this molecular part by quaternary carbons on both sides. This was in agreement with the assignment of two carbonyl functions at δ187.2 and 188.2 for C-1 and C-4, respectively. The α-position of C-14 was concluded from optical rotation ($\alpha = +142.5°$) and in comparison to the literature data of β-oriented bisabolons [16], which were reported to be levorotatory.

The HPLC fraction at R_t 1.43 (relative to DMP) afforded compound **2** with $[M]^+$ at m/z 244 for $C_{15}H_{16}O_3$. ^1H NMR signals were identical with those of compound **1** for the quinone part and the aldehyde function, including the methyl signal for H-12 and the olefinic proton of H-10. However, the sequence of H-7 to H-9 had changed. An additional olefinic signal occurred at δ5.17. Irradiation indicated coupling with two protons at δ2.62 and 2.16, which were assigned on the basis of

Fig. 1. Photomicrograph of a noncapitate glandular trichome from the leaf surface of sunflower (bar equals 10 μm).



1

2 3

additional decoupling with H-10 to H-9a and H-9b. Since no further olefinic proton was present, C-7 had to be quaternary. Further confirmation for the position of the C=C double bond between C-8 and C-7 was given by the mass spectral data, which showed a major fragment at m/z 161 for $[M-83]^+$ in accord with the loss of the C_4H_6CHO moiety.

The IR spectrum of compound **3** showed a strong hydroxyl signal at 3400 cm^{-1}. At the same time the carbonyl signal had decreased relative to the signal observed for compound **1**. This indicated replacement of a carbonyl function by a hydroxyl group, which was confirmed by a signal at δ4.20 in the ^{1}H NMR spectrum, and the fragmentation pattern in the mass spectrum ($[M]^+$ at m/z 248, $C_{15}H_{20}O_3$) was in accord with the loss of water (m/z 230 for $[M-H_2O]^+$). Most of the ^{1}H NMR data were very similar to those of compound **1**. However, the multiplicities of H-2 and H-5 had changed to singlets.

Table 1. ^{1}H NMR data of compounds **1–3** (250 MHz in $CDCl_3$, TMS as int. standard)

H	1	2	3
1	—	—	4.20 *br d*
2	6.61 *q*	6.60 *q*	6.50 *s*
5	6.54 *d*	6.54 *d*	6.45 *br s*
7	2.97 *br q*	—	3.01 *br q*
8a	1.66 *m*[a]	5.19 *dq*	1.66 *m*[a]
8b	1.75 *m*[a]		1.72 *m*[a]
9a	2.34 *dddd*[b]	2.62 *ddq*	2.22 *m*[b]
9b		2.16 *m*	
10	6.45 *ddq*	6.36 *ddq*	6.40 *ddq*
12	1.71 *br d*[c]	1.71 *d*	1.59 *br s*[c]
13	9.38 *s*	9.34 *s*	9.28 *s*
14	1.17 *d*[c]	*	1.16 *s*[c]
15	2.05 *d*[c]	2.02 *d*[c]	2.10 *br s*[c]

[a]Assignment interchangeable. [b]Two-proton signal. [c]Three-proton signal.

*Signal overlapped.

J (Hz): compound **1**: 2,15 = 1.5; 5,7 = 1.0; 7,14 = 7.0; 9a,b = 14; 9a/b,10 = 7.3; 10,12 = 1.2; compound **2**: 2,15 = 1.6; 8,9 = 6.6; 9a,b = 14.1; 9,10 = 7.1; 10,12 = 1.1; compound **3**: 1,2 = 4.5; 5,7 = 1.0; 7,8a = 6.9; 9a/b,10 = 6.1; 10,12 = 1.2.

Table 2. ^{13}CNMR data of compound **1** (100 MHz in $CDCl_3$, TMS as int. standard)

C	1
1	187.2 *s*[a]
2	131.4 *d*[b]
3	145.4 *s*[c]
4	188.2 *s*[a]
5	133.8 *d*[b]
6	139.8 *s*[c]
7	31.3 *d*
8	26.8 *t*
9	34.4 *t*
10	153.2 *d*
11	153.2 *s*
12	19.1 *q*
13	195.1 *d*
14	9.2 *q*
15	15.4 *q*

[a]–[c]Assignment interchangeable.

Since the methyl signal of H-15 was nearly unaffected we assigned the position of the hydroxyl group to C-1 rather than to C-4. The stereochemistry of the methyl group at C-7 was concluded in analogy to compound **1** on the basis of biogenetical reasoning.

Compounds **1–3** were tested for biological activity. All three samples showed cytostatic effects in agar diffusion tests on *Bacillus brevis* (**2**, MIC 180 μg/ml; **1** and **3**, MIC 200 μg/ml). However, the inhibition was about 10 times weaker than that of sesquiterpene lactones of the sunflower [2], which usually react via Michael addition with proteins. To test the thiol alkylating property of the sesquiterpenes reported here, small amounts of compounds **1–3** were incubated with a 10-fold excess of L-cysteine (15 μM sesquiterpene with 150 μM cysteine in MeOH–H_2O, 3:2) for one hour before analysing each sample by HPLC. While chromatographic separation of compound **3** was unaffected, migration of compounds **1** and **2** was accelerated (from R_t 1.73 to R_t 0.60 and from R_t 1.43 to R_t 0.54, respectively), thus indicating an interaction with the amino acid. L-Glycine was used as control and, in contrast, did not influence the retention times of any of the compounds. However, incubation of compound **1** with ascorbate under the same conditions led to a similar accelerated HPLC peak as with cysteine. Measurement of the UV spectrum of this derivative showed two maxima at 230 and 290 nm (as in compound **3**), instead of the original broadly shouldered absorption at 235, 252 (sh) and 262 (sh) nm. Although the small amounts of the purified compounds did not allow additional NMR spectroscopic investigation of the reaction products, these results suggest that cysteine reduced the quinones rather than undergoing a thiol reaction.

Sesquiterpenes, in contrast to the lactonized relatives and to diterpenes, have only twice been reported from *Helianthus* species [20, 21]. Bisabolen-type compounds, while so far unknown from Helianthinae, have previously been isolated from *Coreopsis* [17, 18] and occasionally from species of the tribes Mutisiae [22] and Eupatoriae [19].

EXPERIMENTAL

NMR spectra were measured at 250 and 100 MHz in $CDCl_3$ with TMS as int. standard. MS were recorded by EIMS at 70 eV ionization. IR spectra were performed in $CHCl_3$ and neat on KBr windows. The HPLC separation was performed on Hypersil ODS (5 μm Shandon; analyt. column 250 × 4 mm; prep. column 250 × 20 mm; MeOH–H_2O, 3:2; peak detection at $A_{225\,nm}$).

Isolation of the constituents. The tissue of the intercostal area of 300 g air-dried sunflower leaves (*H. annuus* L. cv giganteus; Fa. Benary, Han.-Münden, Germany; voucher in the herbarium of the University of Tübingen) was carefully removed in order to avoid extraction of major amounts of sesquiterpene lactones. The remaining leaf veins (*ca* 100 g) were rinsed with 500 ml MeOH for 10 min. The sample was filtered, dried under red. pres. and redissolved in MeOH. After addition of H_2O (1:1) the sample was chilled (15 min −20°) and insoluble material was removed by centrifugation (48 000 *g*, 20 min; Beckmann JA 20). The supernatant was evapd *in vacuo* to remove MeOH and the remaining H_2O extract, after filtration, was partitioned against Et_2O. The Et_2O extract was dried under red. pres. and the residue was redissolved in MeOH and diluted with H_2O (final concentration of MeOH–H_2O, 3:2). The sample was filtered over C_{18} cartridges (SEP-PAK; Waters, Milford, U.S.A.) and was then applied on prep. HPLC. Analogous fractions of several HPLC runs were combined and samples were monitored by analytical HPLC to check purity and to identify compounds of the noncapitate glandular trichomes from other constituents of the extract. The major fraction (R_t 1.73 relative to DMP) gave 5.7 mg of **1**. The more polar HPLC fractions afforded 1 mg of **2** and 1.7 mg of **3**.

Glandulone A (**1**). Brownish oil. $C_{15}H_{18}O_3$; EIMS (70 eV) *m/z* (rel. int.): 246 $[M]^+$ (14), 151 $[M-C_5H_6CHO]^+$ (100), $[C_5H_6CHO]^+$ (98), $[C_3H_4CHO]^+$ (34); IR $\nu_{max}^{CH_2Cl}$ cm^{-1}: 1673 (C=C–CHO), 1651 (C=C–CO); $[\alpha]_D^{25}$ +142.5° (MeOH; *c* 0.12); UV λ_{max}^{MeOH} nm: 232, 252 (sh), 262 (sh).

Glandulone B (**2**). Brownish oil. $C_{15}H_{16}O_3$; EIMS (70 eV) *m/z* (rel. int.): 244 $[M]^+$ (13), 161 $[M-C_4H_6CHO]^+$ (100), $[C_3H_4CHO]^+$ (35); IR $\nu_{max}^{CH_2Cl}$ cm^{-1}: 1680 (C=C–CHO), 1652 (C=C–CO); $[\alpha]_D^{25}$ −3.3° (MeOH; *c* 0.12); UV λ_{max}^{MeOH} nm: 235, 253 (sh), 263 (sh).

Glandulone C (**3**). Brownish oil. $C_{15}H_{20}O_3$; EIMS (70 eV) *m/z* (rel. int.): 248 $[M]^+$ (11), 230 $[M-H_2O]^+$ (1), 151 $[M-C_5H_6CHO]^+$ (100); IR $\nu_{max}^{CH_2Cl}$ cm^{-1}: 3409 (OH), 1654 (C=C–CHO); $[\alpha]_D^{25}$ +23.6° (MeOH; *c* 0.12); UV λ_{max}^{MeOH} nm: 230, 292.

Analysis of glands. Noncapitate glandular trichomes were collected from leaf veins with an insect needle or a fine pair of tweezers under a dissection microscope and extracted in solvent. Fifty trichomes were necessary to establish the HPLC pattern of a sample and 300 were applied on TLC analysis.

Bioassay. Determination of the minimum inhibitory concentration (MIC) in antimicrobial testing was carried out in a standard agar diffusion test with *Bacillus brevis*, Migula ATCC 9999, according to ref. [23].

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