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ANNUITHRIN, A NEW BIOLOGICALLY ACTIVE GERMACRANOLIDE FROM *HELIANTHUS ANNUUS*

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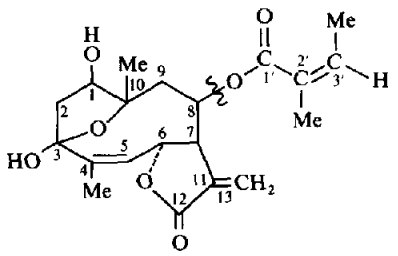
Key Word Index—*Helianthus annuus;* Compositae; ^{1}H NMR and ^{13}C NMR spectra; sesquiterpene lactone; biological activities.

Abstract—Investigations on growth inhibition in *Helianthus annuus* led to the isolation of a new sesquiterpene lactone, a germacranolide with an α-methylene-γ-lactone moiety. The structure of this new germacranolide, annuithrin, was elucidated by spectroscopic methods. Its biological activity has been proven by growth inhibition in straight growth tests, antibacterial tests and inhibition of DNA-/RNA-synthesis in cells of the ascitic form of Ehrlich carcinoma.

INTRODUCTION

Among the angiosperms, particularly the Compositae, are found a great variety of sesquiterpene lactones which are known to have a broad spectrum of biological activities [1–3].Two members of the genus *Helianthus* contain sesquiterpene lactones which inhibit plant growth [4]. *Helianthus tuberosus* contains heliangine [5–7], and *H. annuus* contains a sesquiterpene lactone of unknown structure [4]. In this study, a growth inhibitor from young leaves and the upper part of the stem of *H. annuus* has been shown to be a new heliangolide, which we have named annuithrin (1).



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RESULTS AND DISCUSSION

The structure proposed for annuithrin (1) is based on IR, ^{1}H NMR, ^{13}C NMR, and MS measurements. In accordance with the molecular formula, $C_{20}H_{26}O_7$, the mass spectrum contained a molecular ion at *m/z* 378, and the IR spectrum two carbonyl absorptions at 1750 and 1710 cm^{-1} and a medium strong band at 1650 cm^{-1} for a C=C conjugated to a carbonyl function. The presence of an α-methylene-γ-lactone was indicated by a characteristic pair of doublets for H-13 and H-13′ at δ 6.26 (1H, $J_{7,13}$ = 2.7 Hz) and 5.62 (1H, $J_{7,13'}$ = 2.4 Hz) (Table 1). Irradiation of either of these signals caused a change in the broad, featureless pattern of a three-proton multiplet at δ 4.17. Irradiation at this frequency collapsed the signals at δ 6.26 (H-13) and 5.62 (H-13′) to singlets, whereas a doublet of doublet of doublets at δ 5.57 collapsed to a doublet of doublets, and a doublet of doublets at δ 5.48 collapsed to a doublet. These results clearly indicated that one of the three protons at δ 4.17 must be assigned to H-7, and that the signals at δ 5.57 and 5.48 were due to H-8 and H-6 respectively [8]. Further decoupling at H-6 collapsed a doublet of quartets at δ 5.83 to a singlet indicating a vinylic proton at C-5. By irradiation at this frequency, the signal of H-6 collapsed to a doublet and a three-proton broadened singlet at δ 1.87 was sharpened. These data were consistent with a *trans*-diaxial relationship between the protons at C-5, C-6 and C-7, and revealed that the lactone moiety was cleaved at C-6 and C-7 [9]. The signal of Me-15 at δ 1.87 (3H, *s*) was broadened by allyl coupling,

Table 1. ^{1}H NMR spectral data of 1 (250 MHz, $CDCl_3$, TMS as internal standard)

H	δ	H	δ
1	4.17 (*br*)*	9α	1.98 *dd*
1-OH	4.17 (*br*)*	9β	2.01 *dd*
2α	2.27 *m*	13	6.26 *d*
2β	2.15 *m*	13′	5.62 *d*
3-OH	3.55 *s*(*br*)	14	1.52 *s*
5	5.83 *dq*	15	1.87 *s*(*br*)
6	5.48 *dd*	3′	6.04 *qq*
7	4.17 (*br*)*	4′	1.89 *dq*
8	5.57 *ddd*	5′	1.73 *dq*

J(Hz): 5,6 = 4.3; 5,15 = 0.9; 6,7 = 5.5; 7,8 = 1.5; 7,13 = 2.7; 7,13′ = 2.4; 8,9α = 9.3; 8,9β = 5.3; 9α,9β = 14; 3′,4′ = 7.3; 3′,5′ = 1.5; 4′,5′ = 1.5.

* Broad featureless multiplet.

thereby indicating the presence of a vinyl-methyl group. This coupling was found in the signal of H-5, demonstrating that Me-15 and H-5 were *cis*-orientated as in related compounds [8, 10]. Decoupling at H-8 changed the pattern of the three-proton multiplet at δ 4.17 and collapsed two doublets of doublets at δ 1.98 and 2.01 to two doublets, thus giving the frequency of H-9α and H-9β. The shift value and the coupling pattern of H-8 showed that this carbon atom had a side chain attached to it [9]. Irradiation at the centre of a doublet of quartets at δ 1.89 collapsed a doublet of quartets at δ 1.73 to a singlet and a quartet of quartets at δ 6.04 to a singlet, revealing the presence of an angilic acid moiety cleaved at C-8 [11]. From biogenetic consideration, it was assumed that H-7 was in the α-position [6]. The small value of $J_{7,8}$ suggested a dihedral angle of about 120° relating these two protons and placing H-8 in the α-position. This was confirmed by the lack of a geminal coupling and the shift of H-13 [12]. The unusual low chemical shift of H-7 indicated a very narrow neighbourhood to an oxygen atom, but further ^{1}H-decoupling experiments did not clarify this point.

^{13}C-broadband and off-resonance decoupled spectra performed in a special microcell gave further information. Two signals at δ 106.3 and 83.4 (both singlets in the off-resonance decoupled spectrum) (Table 2) revealed the existence of a hemiacetal linkage. Construction of a Dreiding model clearly showed that the hemiacetal linkage was between C-10 and C-3. They also indicated that the C-4 Me group and H-5 were *cis*. In the ^{1}H NMR spectra the presence of the hemiacetal moiety located at C-3 and C-10 was given by the H-14 signal at δ 1.52 (3H, *s*) and the OH resonance at δ 3.55 [1H, *s*(*br*)]. By addition of D_2O the signal of the C-3 hydroxyl group disappeared and a change in the featureless multiplet at δ 4.17 was observed. The last remaining proton, therefore, was assigned to a hydroxyl group attached to either C-1 or C-2. Because of the difficulties in analysing the complex pattern of the three-proton multiplet at δ 4.17, the position of the remaining hydroxyl group was only indicated by comparison with data for similar compounds. The ^{1}H NMR and ^{13}C NMR spectral data of annuithrin were very similar to those of tagitinin A [10] and a new sesquiterpene lactone isolated from *Calea* [12]. On biogenetic grounds, it was assumed that the remaining hydroxyl group was located at C-1 (this was also indicated by ^{13}C NMR shifts). With the help of the chemical shifts of tagitinin A and the *Calea* sesquiterpene lactone, we were able to assign the complex one-proton multiplets at δ 2.27 and 2.15 to H-2α and H-2β. In *Calea* sesquiterpene lactone the C-1 hydroxyl group is β-orientated and thus causes deshielding of H-9β. In annuithrin, we did not observe such a chemical shift. Therefore, we tentatively proposed that the C-1 hydroxyl group was α-orientated. By careful investigation of other compounds isolated in our laboratory from *H. annuus*, we intend to clarify this stereochemical problem.

Table 2. ^{13}C NMR spectral data of **1** (22.63 MHz, $CDCl_3$, TMS as internal standard)

C	δ	C	δ
1	77.3 *d*	11	143.1 *s*
2	37.4 *t*	12	169.8 *s*
3	106.3 *s*	13	123.4 *t*
4	136.4 *s*	14	20.4 *q*
5	132.6 *d*	15	20.3 *q*
6	74.9 *d*	1′	166.6 *s*
7	50.0 *d*	2′	127.1 *s*
8	72.0 *d*	3′	139.6 *d*
9	41.0 *t*	4′	15.7 *q*
10	83.4 *s*	5′	20.3 *q*

All the data were consistent with the structure proposed for annuithrin (**1**). The mass spectrum exhibited a strong peak at *m/z* 378 (relative intensity 19.3%) for the molecular ion and peaks at *m/z* 360 (9.1%) and 342 (2.7%) for the stepwise elimination of two molecules of water. The fragmentation of the ester linkage gave either the stable acyl ion at *m/z* 100 (23.6%) or the ion at *m/z* 278 (8.2%). The occurrence of the fragment at *m/z* 100 was in accordance with the presence of an ester of angilic acid. The formation of the ion at *m/z* 278 was followed by the loss of two molecules of water (*m/z* 260, 242). The sequence 279, 261, 243 was accompanied by three ions at *m/z* 278,260 and 242. This fragmentation pathway started with a McLafferty-like rearrangement to *m/z* 278 followed by successive elimination of two water molecules.

Biological activities

Annuithrin was tested in the *Avena* straight growth test. Addition in a concentration range from 50 to 180 μM resulted in a linear reduction of growth between 10 and 90%. Growth assays on *Avena* and *Helianthus* with an inductive displacement transducer show that annuithrin inhibited the IAA-induced straight growth of stem segments from the *H. annuus* analogue to that of *Avena* coleoptile sections. This result indicated that annuithrin may be an endogenous growth substance in sunflower. The chemical structure of this substance with the exocyclic methylene group conjugated to a γ-lactone and the functional groups predict other biological activities [1]. In fact, annuithrin was shown to have antibacterial qualities. However, fungi and yeast were either less inhibited or not inhibited (MIC 45 μg/ml on *Bacillus brevis*; MIC 90 μg/ml on *Proteus vulgaris*; MIC 90 μg/ml on *Eremothecium ashbyi*). *In vivo* DNA and RNA synthesis in cells of the ascitic form of Ehrlich carcinoma was drastically reduced by annuithrin (at an annuithrin concentration of 20 μg/ml about 50% inhibition of DNA synthesis and about 75% inhibition of RNA synthesis) (T. Anke *et al.*, unpublished results). More detailed results and further biological investigations are to be the subject of a further report.

EXPERIMENTAL

Plant material. Helianthus annuus var. giganteus was grown in the greenhouse.

Extraction and isolation. Young leaves and stems of 3-week-old plants were extracted in boiling EtOH, homogenized and filtered. The filtrate was evapd *in vacuo* and the residue extracted by Et_2O. The crude extract was chromatographed by SC (Polygosil 60-4063) and further purified by HPLC (Nucleosil RP 18, 10 μm, 250 × 4.8 mm, Waters M 6000, Reodyne 7125). The inhibitor fractions were analysed by bioassay.

Annuithrin (**1**). Light yellow, thick oil. Yield: 43.5 ± 6.3 (SEM, $n = 4$) μg/g fr. wt. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3570 (OH), 1760 (OCOR),

1710 (ester), 1650 (C=C). MS 70 eV *m/z* (rel. int.): 378 (M^+, 19.3), 360 ($M - H_2O$, 9.1), 342 ($M - 2H_2O$, 2.7), 279 ($M - C_5H_7O_2$, 4.9), 278 ($M - C_5H_8O_2$, 8.3), 261 (279 − H_2O, 17.5), 260 (278 − H_2O, 36.7), 243 (261 − H_2O, 14), 242 (260 − H_2O, 17), 100 ($C_5H_8O_2$, 23.6), 99 ($C_5H_7O_2$, 32.2), 83 (C_5H_7O, 100). (Calc. for $C_{20}H_{26}O_7$: 378.4212, found: (MS) 378). CD (MeOH): λ_{max} 235 nm; $[\theta]_{225} - 40 \times 10^2$, $[\theta]_{235}$ 37.5 $\times 10^2$, $[\theta]_{260}$ 21.5 $\times 10^2$, $[\theta]_{280}$ 5 $\times 10^2$; UV (EtOH): strong end absorption, $\varepsilon = 21\,000$ at 210 nm.

Bioassay (Avena *straight growth test*). Etiolated *Avena* coleoptile sections (10 mm long, apical 2 mm sections discarded) were incubated in 5 mM MES buffer, pH 5.85, for 4 hr at 30°. Each assay tube contained 5 ml of test soln (buffer plus 10 mM IAA plus annuithrin in different concns) and ten coleoptile segments. The growth assay with the inductive displacement transducer was performed by a Philips PR 9314-01 registration unit in the same test soln. The determination of the minimum inhibitory concn (MIC) in antimicrobial testing was carried out as described by Drews [13]. Inhibition of *in vivo* DNA and RNA synthesis in cells of the ascitic form of Ehrlich carcinoma was investigated according to refs. [14] and [15].

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REFERENCES

1. Rodriguez, E., Towers, G. H. N. and Mitchell, J. C. (1976) *Phytochemistry* **15,** 1573.
2. Rücker, G. (1973) *Angew. Chemie* **85,** 895.
3. Wagner, H. and Wolff, P. (eds.) (1977) *New Natural Products and Plant Drugs with Pharmacological, Biological or Therapeutical Activity.* Springer, Berlin.
4. Shibaoka, H. (1961) *Plant Cell Physiol.* **2,** 175.
5. Morimoto, H., Sanno, Y. and Oshio, H. (1966) *Tetrahedron* **22,** 3174.
6. Neidle, S. and Rogers, D. (1972) *J. Chem. Soc. Chem. Commun.* 140.
7. Nishikawa, M., Kamiya, K., Takabatake, A., Oshio, H., Tomiie, Y. and Nitta, J. (1966) *Tetrahedron* **22,** 3601.
8. Herz, W. and Bhat, S. V. (1972) *J. Org. Chem.* **37,** 906.
9. Lee, K.-H., Kimura, T., Okamoto, M. and Cowherd, C. M. (1976) *Tetrahedron Letters* 1051.
10. Baruah, N. C., Sharma, R. P., Madhusudan, K. P., Thyagarajan, G., Herz, W. and Murari, R. (1979) *J. Org. Chem.* **44,** 1831.
11. Rustaiyan, A., Nazarians, L. and Bohlmann, F. (1980) *Phytochemistry* **19,** 1230.
12. Bohlmann, F. and Zdero, C. (1977) *Phytochemistry* **16,** 1065.
13. Drews, G. (1976) *Mikrobiologisches Praktikum.* Springer, Berlin.
14. Weitzel, G., Schneider, F., Fretzdorff, A. M., Durst, J. and Hirschmann, W. D. (1967) *Hoppe-Seyler's Z. Physiol. Chem.* **348,** 433.
15. Martin, E. M., Malec, J., Sved, S. and Work, T. S. (1961) *Biochem. J.* **80,** 585.

NOTE ADDED IN PROOF

During preparation and after acceptance of our manuscript, Ohno, N. and Mabry, T. J. [(1980) *Phytochemistry* **19,** 609] as well as Herz, W. and Kumar, N. [(1981) *Phytochemistry* **20,** 93] independently reported the isolation of a compound from *Helianthus niveus* (structure **7**) and *Helianthus maximiliani* (structure **4a**), which is identical with our annuithrin from *Helianthus annuus* in most of the published dates.