Herbarulide, a Ketodivinyllactone Steroid with an Unprecedented Homo-6-oxaergostane Skeleton from the Endophytic Fungus *Pleospora herbarum*[1]

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The novel ketodivinyllactonic steroid, herbarulide (**1**), deviating from the brassinosteroids in the position of the oxygen, was isolated from the endophytic fungus *Pleospora herbarum*. The structure was determined by extensive NMR studies, including particularly HMQC and HMBC experiments.

As part of a program on the isolation of biologically active compounds as leads for new products for pharmacy and for plant protection, we have been investigating the secondary metabolites produced by endophytic fungi. These relatively unexplored fungi have proved to be a rich source of novel secondary metabolites.[2] For example, the palmarumycines, a new class of naphthalene spiroketals, were isolated from the endophytic fungus *Coniothyrium palmarum*.[3,4] We now report on the investigation of constituents of the ascomycete *Pleospora herbarum* (order Dothidiales), an endophyte of *Medicago lupulina*. The fungus was cultivated on biomalt or soja-malt soft agar. The crude fermentation extracts showed algicidal (*Chlorella fusca*), herbicidal, and moderate antimicrobial activity against selected fungi and bacteria. The interleukin-1β converting enzyme was also inhibited by the extract.

Four pure compounds were isolated after extensive chromatography of an extract. Two of these, ergosta-4,6,8-(14),22-tetraen-3-one,[5] and stemphyperylenol,[6] were known compounds. The data, with the exception of the opposite optical rotation of the second most polar compound, were identical with those of probetaenone I,[7] and the compound must be the mirror image *ent*-probetaenone I. The second new natural product, named herbarulide (**1**), was shown to have a steroidal skeleton with an unprecedented oxygen ring, closely related to the major constituent ergosta-4,6,8-(14),22-tetraen-3-one. The interesting ketodivinyllactone structural element of **1** was heretofore unknown in steroids. The well-known brassinosteroids, which are important plant growth regulators,[8] also have a lactone group in the seven-membered steroidal ring B. However, the spectral data indicated that the position of the oxygens in ring B was not identical to that of the brassinosteroids, as shown in the structure of brassinolide (**2**). Therefore, our attention was focused on unambiguous location of the oxygens and double bonds in **1**.

The molecular formula of herbarulide (**1**) was $C_{28}H_{40}O_3$, as determined by HREIMS (*m/z* 424.293). IR signals at 1732 and 1682 cm^{-1} indicated the presence of an ester and an α,β-unsaturated ketone. The ^{1}H and ^{13}C NMR spectra showed a typical steroidal pattern, closely related to ergosta-4,6,8(14),22-tetraen-3-one. The ^{13}C and ^{13}C-DEPT NMR data allowed identification of six quaternary carbons, 10 methines, six methylenes, and six methyl groups. The resonances of the corresponding protons were assigned by an HMQC spectrum. The assignments based on the H−H COSY and HMQC spectra are summerized in Table 1. The most important HMBC correlations are shown in Figure 1.

Table 1. ^{1}H and ^{13}C NMR Data for Herbarulide (**1**)[a]

position	δ_H	δ_C	COSY	HMBC
1_a	2.19 m	34.0 t	H-1_b, H-2	C-2, 3, 9, 10, 19
1_b	1.95 m	34.0 t	H-1_a, H-2	C-2, 3, 9, 10, 19
$2_{a,b}$	2.46 m	33.2 t	H-$1_{a,b}$	C-3, 10
3		198.4 s		
4	5.75 s	114.7 d		C-5
5		173.9 s		
6		162.4 s		
7	5.72 s	113.4 d		C-6, 8
8		159.4 s		
9	2.52 m	47.3 d	H-$11_{a,b}$	C-1, 7, 10, 8, 11, 19
10		40.4 s		
11_a	1.84 m	25.4 t	H-9, 11_b, $12_{a,b}$	
11_b	1.68 m	25.4 t	H-9, 11_a,$12_{a,b}$	
12_a	2.09 m	39.2 t	H-$11_{a,b}$, 12_b	C-11, 18
12_b	1.45 m	39.2 t	H-$11_{a,b}$, 12_a	C-11, 18
13		47.0 s		
14	2.14 m	58.2 d	H-15	C-7, 8, 13, 15, 18
15	1.50 m	22.6 t	H-14, 16	
16	1.78 m	27.7 t	H-15, 17	
17	1.38 m	56.4 d	H-16, 20	C-16, 20
18	0.64 s	12.5 q		C-12, 13, 14, 17
19	1.24 s	20.0 q		C-1, 5, 9, 10
20	2.03 m	40.2 d	H-17, 21, 22	C-17, 22, 23
21	1.02 d 6.6	21.1 q	H-20	C-17, 20, 22
22	5.15 dd 15.2, 8.5	134.7 d	H-20, 23	C-23
23	5.25 dd 15.2, 7.8	132.9 d	H-22, 24	C-22
24	1.86 m	42.9 d	H-23, 25, 28	C-22, 23, 25, 28
25	1.48 m	33.1 d	H-24, 26, 27	C-23, 24, 28
26	0.84 d 6.8	20.0 q	H-25	C-24, 25
27	0.82 d 6.8	19.7 q	H-25	C-24, 25
28	0.91 d 6.8	17.6 q	H-24	C-23, 24, 25

[a] Data for **1** were recorded in $CDCl_3$ at 600 MHz (^{1}H) and 150 MHz (^{13}C). All assignments were confirmed by DEPT experiments; s = quaternary, d = tertiary, t = secondary, q = primary atoms.

The HMBC correlations of the H-18 methyl protons to the adjacent carbon atoms C-12, C-13, C-14, and C-17 defined the nature of these carbons, because, of these, C-13 was the only quaternary carbon. The arrangement of C-1, C-5, C-9, and C-10, neighboring C-19, was fixed as shown in Figure 1 for similar reasons. The ^{13}C NMR chemical shifts C-7 (δ 113.4) and C-8 (δ 159.4) indicated that they were part of an α,β-unsaturated carbonyl group, with C-7 in the α-position and C-8 in the β-position. C-6 was

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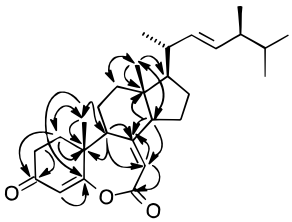
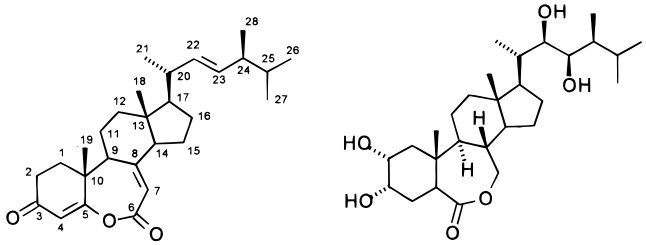


Figure 1.

identified as an ester carbonyl atom connected to C-7, because it showed a ^{13}C NMR chemical shift of δ 162.4 with an HMBC correlation to H-7. This α,β-unsaturated carbonyl element was located in the molecular skeleton by the HMBC correlations of H-9 and H-14 to C-7 and C-8. The only remaining olefinic bonding partner of C-4 was C-5. Furthermore, C-5 must be connected to the sp^3 ester oxygen atom, because of its extremely lowfield ^{13}C NMR (δ 173.9). Only C-2 was left for the keto group, which was also required by HMBC correlations of C-3 to H-1 and H-2 and the IR signal at 1682 cm^{-1} for an α,β-unsaturated ketone. The structural characteristics of **1** as deduced from these data are demonstrated by comparison with brassinolide (**2**).



herbarulide (**1**) brassinolide (**2**)

The stereochemistry of the side chain then needed to be determined. The coupling constant (J = 15.2 Hz) between H-22 and H-23 indicated an *E* configuration of the carbon−carbon double bond at C-22. The stereochemistry at C-20 and C-24 was assumed to be identical to that of (20*R*,24*S*)-ergosta-4,6,8(14),22-tetraen-3-one[5] as suggested by the nearly identical NMR spectra (particularly the ^{13}C NMR chemical shifts) in the side chain of the two molecules.

Experimental Section

General Experimental Procedures. For organism and instrumentation, see Krohn et al.[3]

Fermentation of *Pleospora herbarum*, Extraction, and Isolation of Metabolites. The fungus was cultivated on a biomalt (5% w/v) or soja-malt (30 g/L malt extract, 3 g/L soja flour, pH 5.5) semisolid agar for 48 days. The fungal culture, including agar medium and fungal mycelium, was homogenated in a Waring blender. The homogenate was extracted three times with petroleum ether (500 mL) and EtOAc (500 mL). The extracts showed no major differences on TLC and were combined and evaporated under vacuum to yield 4.4 g of crude extract. The crude extract was separated into four fractions by chromatography on Si gel using gradients of increasing polarity of CH_2Cl_2 and MeOH (100:0 to 4:1). The crude nonpolar fractions were then further purified by TLC (1 mm of Si gel) to yield pure (20*R*,24*S*)-ergosta-4,6,8(14),22-tetraen-3-one (11 mg; R_f 0.25, CH_2Cl_2), herbarulide (**1**) (3.7 mg; R_f 0.28, CH_2Cl_2), *ent*-probetaenone I (5.3 mg; R_f 0.29, CH_2Cl_2). The most polar fraction was further purified by column chromatography using Lobar Si gel columns (Merck) to afford stemphyperylenol (6.2 mg; R_f 0.28, CH_2Cl_2−5% MeOH). For data for (20*R*,24*S*)-ergosta-4,6,8(14),22-tetraen-3-one, *ent*-probetaenone I, and stemphyperylenol, see supporting material.

10a,12a-Dimethyl-1-(1,4,5-trimethyl-hex-(*E*)-2-enyl)-2,3,3a,9,10,10a,10b,11,12,12a-decahydro-1*H*-6-oxa-benzo-[3,4]cyclohepta[1,2-*e*]indene-5,8-dione (2): colorless oil, $[\alpha]^{25}_D$ +55° (*c* 0.185, CH_2Cl_2); UV (MeOH) λ_{max} (log ϵ) 277 (2.76) nm; IR (film) ν_{max} 2954, 1732, 1682, 1622, 1454, 1385, 1261, 1138, 1036 cm^{-1}; ^1H NMR, see Table 1; ^{13}C NMR, see Table 1; EIMS *m/z* 424 (51) [M^+], 396 (100), 381 (25), 341 (22), 299 (30), 298 (38), 297 (41), 269 (22), 255 (21), 173 (19), 145 (19), 127 (39), 126 (66), 86 (55), 69 (57), 55 (52); HREIMS *m/z* 424.293 (calcd for $C_{28}H_{40}O_3$, 424.297).

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Supporting Information Available: For spectroscopic data of (20*R*,24*S*)-ergosta-4,6,8(14),22-tetraen-3-one, *ent*-probetaenone I, and stemphyperylenol. This material is available free of charge via the Internet at http://pubs.acs.org.

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