# Secondary Metabolites by Chemical Screening. 30.[1] Helmidiol, a New Macrodiolide from *Alternaria alternata*

Rüdiger Kind,[†] Axel Zeeck,*[,†] Susanne Grabley,[‡] Ralf Thiericke,*[,‡] and Marion Zerlin[‡]

*Institut für Organische Chemie, Universität Göttingen, Tammannstrasse 2, D-37077 Göttingen, Germany, and Hans-Knöll-Institut für Naturstoff-Forschung e.V., Beutenbergstrasse 11, D-07745 Jena, Germany*

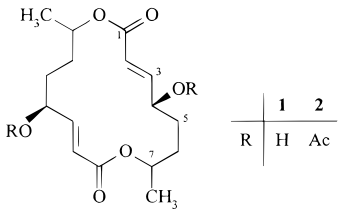
*Received November 21, 1995*[⊗]

Chemical screening of *Fungi imperfecti* strains resulted in the detection, isolation, and structure elucidation of a new symmetric 16-membered macrodiolide, named helmidiol (**1**). This secondary metabolite from *Alternaria alternata* (strain FH-A 6965) shows anthelmintic properties.

Application of our chemical screening methodology[1,2] to different *Fungi imperfecti* isolates[3,4] gave rise to the detection, isolation, structure elucidation, and broad biological testing of a new secondary metabolite named helmidiol (**1**), a symmetric 16-membered macrodiolide.

The helmidiol-producing strain FH-A 6965 was isolated from the surface of *Adesmia boronioides* (Fabaceae) collected in Argentina. From various morphological and chemotaxonomical investigations the strain was classified as *Alternaria alternata* (Hyphomycetes),[5] showing a yellow to brown mycelium on medium A. In our screening routine the strain was cultivated for five days at 25 °C in 300-mL Erlenmeyer flasks containing 100 mL of medium B. Using the already described work-up procedure we obtained a defined extract of the culture filtrate, which was subjected to a TLC analysis in different solvent systems and a variety of staining reagents.[1−4] On Si gel TLC plates [$R_f$ values: 0.92 (EtOAc−MeOH−$H_2O$, 6:2:1), 0.68 ($CHCl_3$−MeOH, 9:1), 0.29 (EtOAc−*n*-hexane, 4:1), and 0.73 (*n*-BuOH−AcOH−$H_2O$, 4:1:5, upper phase)] helmidiol showed color reactions with various staining reagents, for example, anisaldehyde/$H_2SO_4$ (green-blue), vanillin/$H_2SO_4$ (brown), molybdatophosphoric acid (green-blue), Ehrlich's reagent (white), orcinol (orange-brown), and blue tetrazolium reagent (violet, weak colorization). In order to isolate helmidiol, a fermentation was carried out in a 10-L scale for four days. After harvesting, EtOAc-extraction, followed by subsequent Si gel and Sephadex LH-20 gel permeation chromatography, resulted in the isolation of pure helmidiol (yield: 59 mg/L).

The structure of helmidiol was ascertained by detailed analysis of $^1$H-, $^{13}$C-, and 2D (e.g., $^1$H−$^1$H-COSY and $^1$H−$^{13}$C-HETCOR) NMR spectra in combination with derivatization studies. The molecular formula $C_{16}H_{24}O_6$ of helmidiol resulted from a CIMS and a HREIMS, ($M^+/2$ = 156.1273, $C_8H_{12}O_3$) and, as an indirect proof, from diacetyl helmidiol (**2**) (HREIMS, $M^+$ = 396.1784, $C_{20}H_{28}O_8$), although a FABMS of the native compound gave no molecular ion. Although the $^1$H- and $^{13}$C-NMR spectra presented the signals of only half of the symmetric molecule, the diolide structure was unambiguously proved by the MS data of both **1** and its corresponding diacetate derivative **2**. The positions of the substituents in the carbon chains resulted from the cross peaks in the $^1$H−$^1$H-COSY NMR experiment. From the coupling constants ($J_{2\text{-H},3\text{-H}}$ = 15.7 Hz) it was deduced that the double bonds are trans, while their location was ascertained from the chemical shifts in the $^{13}$C-NMR spectrum (proton connectivity was deduced from the $^1$H$^{13}$C-HETCOR).




| | 1 | 2 |
|---|---|---|
| R | H | Ac |

Diacetyl helmidiol (**2**), obtained by treatment of **1** with acetic anhydride/pyridine, showed the expected downfield shift of H-4 (from $\delta_H$ 4.31 to 5.22) and an additional signal of the acetyl group (OAc-4 $\delta_H$ 2.08). A selective esterification of the hydroxy groups in **1** with 2(*R*)- or 2(*S*)-phenylbutyric acid according to Helmchen's method[6] clarified the absolute stereochemistry at C-4. A comparison of the $^1$H-NMR spectra of the diastereomeric esters revealed the (*S*)-configuration at C-4, indicated by significant highfield shifts of the olefinic protons H-2 ($\Delta$ $\delta_H$ 0.26 ppm) and H-3 ($\Delta$ $\delta_H$ 0.21 ppm) of the diastereomer that derived from 2(*S*)-phenylbutyric acid. Unfortunately, the corresponding shifts of H-5 could not be determined because of the complex signals in the $^1$H-NMR spectra ($\delta_H$ 1.5−1.95 ppm). According to these data the structure of the symmetric 16-membered dilactone helmidiol has been established as depicted in **1**. However, the configuration of C-7 in both halves of the molecule are still unknown.

On the basis of prior work on the macrodiolides elaiophylin[7] and clonostachydiol,[8] the anthelmintic action of helmidiol was investigated in in vivo tests using lambs (30−40 kg body wt) artificially infected with infective stages of abomasum nematodes (*Haemunchus cortortus*). The application of 2.5 mg/kg of **1** subcutaneously produced a ca. 50% reduction of the nematodes as measured by coproscopic investigations before and after 14 days. However, in the cases of helmidiol and clonostachydiol the anthelmintic effect could repeatably be observed in one group of lambs, while in others no effect was found. We attribute these findings to differences in the metabolism of the administrated compounds, but this has not been proved up to now.

The characteristic symmetric diolide structure is seldom observed as a structural element of natural products. However, 16-membered dilactones of the helmidiol type are found in pyrenophorin[9] isolated from

* Authors to whom correspondence should be addressed.
† Institut für Organische Chemie.
‡ Hans-Knöll-Institut.
⊗ Abstract published in *Advance ACS Abstracts,* May 1, 1996.

S0163-3864(96)00083-3 CCC: $12.00 

the phytopathogen fungus *Pyrenophora avenae* and vermiculin[10] from *Penicillium vermiculatum*, which bears a keto group at C-4 (1: OH-4). Whereas vermiculin exhibits an exocyclic ketopropyl side chain, an oxazole side chain is to be found in conglobatin,[11] isolated from *Streptomyces conglobatus*. Additionally, elaiophylin,[7] with two conjugated diene systems in the 16-membered dilactone ring, is a glycosylated (2-desoxy-L-fucose) symmetric macrodiolide. Furthermore, the symmetric dilactone element is observed in the complex marine metabolites swinholide A[12] and misakinolide A[13] isolated from different *Theonella* species.

## Experimental Section

**General Experimental Procedures.** IR spectra on KBr disks were recorded on a Perkin-Elmer Model 297 spectrometer and UV spectra on a Kontron Uvikon 860 instrument. EIMS and CIMS ($NH_3$) were obtained with Varian MAT-731 (70 eV) and Finnigan MAT-311A mass spectrometers, direct insert, and for HRMS high-resolution perfluorokerosine was used as a standard. NMR spectra were measured with a Varian VXR-200 instrument. Chemical shifts are expressed in $\delta$ values with TMS as internal standard. TLC was performed on Si gel plates (Merck, HPTLC plates, $60F_{254}$ on glass) and column chromatography on Si gel 60 (Merck, 0.040 × 0.063 mm) or Sephadex LH-20 (Pharmacia). Fermentation was carried out in a 10-L fermenter from Braun Dissel (Biostat E, Melsungen, Germany).

**Culture Material.** *A. alternata* (strain FH-A 6965, deposited in the German Culture Collection: DSM 7150) was grown on agar slants containing malt extract 2%, yeast extract 0.2%, glucose 1%, $(NH_4)_2HPO_4$ 0.05%, agar 2%, pH 6.0 prior to sterilization (medium A) for 10−14 days at 25 °C. Storage of the strain was carried out in 50% aqueous glycerol at −20 °C.

**Fermentation.** For the production of **1** the glycerol-containing storage mixture (3 mL) was used to inoculate a 500-mL Erlenmeyer flask containing medium A[1,2] omitting agar (medium B, 100 mL). The flask was cultivated on a rotary shaker (140 rpm) for 3−6 days for the primary screening. Cultures, 72-h old, prepared in the same manner were used to inoculate a fermenter (10-L working volume, inoculation volume 5%, 200 rpm, 25 °C, aeration 5 L/min) containing glucose 1%, soluble starch 0.5%, maize starch, 0.5%, yeast extract 0.5%, cornsteep 0.5%, $CaCO_3$ 0.2%, pH 5.5 prior to sterilization (medium C). Usually, highest yields (ca. 59 mg/L) were reached after about 96 h. Foaming could be decreased using EtOH polyol solutions (e.g., Niax polyol). Details of the chemical screening method (cultivation, adsorption of the culture filtrate, concentration steps, and the conditions for TLC analysis) were reported previously.[1−4]

**Isolation.** After harvesting, the culture broth (~10 L) was filtered, and the culture filtrate was extracted three times with 3 L of EtOAc. The combined organic layers were evaporated to dryness. The mycelium was extracted twice with $Me_2CO$ (1 L), evaporated to a watery residue, and lyophylized. Both crude extracts were combined and chromatographed on Si gel (column: 38 × 8 cm; $CHCl_3$−MeOH, 9:1) and Sephadex LH-20 (column: 38 × 8 cm; MeOH). Compound **1** was further purified on Si gel (column: 40 × 4 cm; $Me_2CO$−*n*-hexane, 1:2) to yield 59 mg/L of pure amorphous product.

**Helmidiol (1):** mp 131 °C; $[\alpha]^{22}_D$ −14.9° (*c* 1, $CHCl_3$) and +3.8° (*c* 1, MeOH); UV $\lambda$ max (MeOH) 207 ($\epsilon$ 17850) nm, $\lambda$ max (MeOH + HCl) 206 (18650) nm, $\lambda$ max (MeOH + NaOH) 215 (16040) nm; CD $\lambda$ extreme (MeOH) 220 ($\Theta$ −2.93 $10^5$) nm; IR (KBr) $\nu$ max 2980, 2940, 2865, 1725, 1655, 1458, 1355, 1300, 1280, 1270, 1170 $cm^{-1}$; $^1H$ NMR (200 MHz, $CDCl_3$, signals of one half of the symmetric molecule) $\delta$ 1.28 (3H, d, $J$ = 6.5 Hz, Me-7), 1.69 (2H, m, $H_2$-6), 1.90 (2H, m, $H_2$-5), 2.30 (1H, br s, OH-4), 4.31 (1H, m, H-4), 5.14 (1H, m, H-7), 5.98 (1H, dd, $J$ = 15.7, 1.7 Hz, H-2), 6.91 (1H, dd, $J$ = 15.7, 5.5 Hz, H-3); $^{13}C$ NMR (50.3 MHz, $CDCl_3$, signals of one-half of the symmetric molecule) $\delta$ 18.6 (Me-7), 28.9 (C-5 or C-6), 30.6 (C-5 or C-6), 70.1 (C-4 or C-7), 70.3 (C-4 or C-7), 122.0 (C-2), 149.3 (C-3), 165.6 (C-1); EIMS (*m*/*z* 70 eV) 156.1273 (70, $M^+/2$, $C_8H_{12}O_3$, calcd. 156.1273 and found) 139 (30), 114 (30), 111 (50), 93 (43), 84 (45), 55 (72), 43 (44); CIMS (*m*/*z* $NH_3$) 331 (100, M + $NH_3$ + 2 $H^+$).

**Diacetyl Helmidiol (2).** Compound **1** (16 mg, 0.051 mmol) was dissolved in a mixture of 2 mL of $Ac_2O$ in 5 mL of pyridine and stirred for 2 h at room temperature. After being poured on ice−water, the reaction mixture was extracted three times with 50 mL of EtOAc and purified by chromatography on Si gel (column: 20 × 1.5 cm, $CHCl_3$-MeOH, 9:1) to yield 19 mg (93.6%) of pure amorphous **2**: $^1H$ NMR (200 MHz, $CDCl_3$, signals of one-half of the symmetric molecule) $\delta$ 1.27 (3H, d, $J$ = 6.5 Hz, Me-7), 1.50−1.80 (2H, m, $H_2$-6), 1.80−1.95 (2H, m, $H_2$-5), 2.08 (3H, s, OAc-4), 5.10 (1H, m, H-7), 5.22 (1H, m, H-4), 5.98 (1H, dd, $J$ = 15.7, 1.7 Hz, H-2), 6.80 (1H, dd, $J$ = 15.7, 6.5 Hz, H-3); EIMS (*m*/*z* 70 eV) 396.1784 (0.2, $M^+$, $C_{20}H_{28}O_8$, calcd 396.1784 and found), 354 (0.9), 337 (2.6), 294 (8), 199 (4.6), 155 (17), 139 (73), 93 (22), 55 (19), 43 (100).

**Acknowledgments.** We express our thanks to A. Godawa, M. Oswald, P. Stapf, and K. Spitzenberger for excellent technical assistance, to Dr. J. Wink for microbiological work, and to Drs. D. Düwel and K. Schmid for biological testing. This work was done in the course of a collaboration project between the Hoechst AG and Prof. Dr. A. Zeeck, University of Göttingen, and was granted by the Bundesministerium für Forschung und Technologie (BMFT, grant 0319311B).

## References and Notes

(1) For part 29 of this series, see: Göhrt, A.; Grabley, S.; Thiericke, R.; Zeeck, A. *Liebigs Ann. Chem*. **1996**, 627−633.
(2) Bach, G.; Breiding-Mack, S.; Grabley, S.; Hammann, P.; Hütter, K.; Thiericke, R.; Uhr, H.; Wink, J.; Zeeck, A. *Liebigs Ann. Chem*. **1993**, 241−250.
(3) Grabley, S.; Granzer, E.; Hütter, K.; Ludwig, D.; Mayer, M.; Thiericke, R.; Till, G.; Wink, J. *J. Antibiot*. **1992**, *45*, 56−65.
(4) Göhrt, A.; Zeeck, A.; Hütter, K.; Kirsch, R.; Kluge, H.; Thiericke, R. *J. Antibiot*. **1992**, *45*, 66−73.
(5) van Arx, J. A., Ed. *The genera of Fungi Sporulating in Pure Culture*; Ganter Verlag: Vaduz, 1981.
(6) Helmchen, G. *Tetrahedron* **1974**, *16*, 1527−1530.
(7) Hammann, P.; Kretzschmar, G.; Seibert, G. *J. Antibiot*. **1990**, *43*, 1431−1440.
(8) Grabley, S.; Hammann, P.; Thiericke, R.; Wink, J.; Philipps, S.; Zeeck, A. *J. Antibiot*. **1993**, *46*, 343−345.
(9) Grove, J. F. *J. Chem. Soc. C* **1971**, 2261−2263.
(10) Fuska, J.; Nemec, P.; Kuhr, I. *J. Antibiot*. **1972**, *25*, 208−211.
(11) Westley, J. W.; Liu, C.-M.; Evans, R. H.; Blount, J. F. *J. Antibiot*. **1979**, *32*, 874−877.
(12) Doi, M.; Ishida, T.; Kobayashi, M.; Kitigawa, I. *J. Org. Chem*. **1991**, *56*, 3629−3632.
(13) Tanaka, J.; Higa, T.; Kobayahi, M.; Kitigawa, I. *Chem. Pharm. Bull*. **1990**, *38*, 2967−2970.

NP960083Q