ORGANIC LETTERS

2001
Vol. 3, No. 22
3479−3481

The Structure of Microcarpalide, a Microfilament Disrupting Agent from an Endophytic Fungus

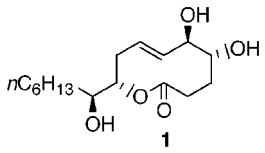
Anokha S. Ratnayake, Wesley Y. Yoshida, Susan L. Mooberry,[†] and Thomas Hemscheidt*

Department of Chemistry, University of Hawaii, 2545 McCarthy Mall, Honolulu, Hawaii 96822, and Natural Products Program, Cancer Research Center of Hawaii, Lauhala Street, Honolulu, Hawaii 96813

tomh@gold.chem.hawaii.edu

Received July 27, 2001

ABSTRACT



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A new alkyl-substituted nonenolide, microcarpalide 1, has been isolated from fermentation broths of an unidentified endophytic fungus. Microcarpalide is weakly cytotoxic to mammalian cells and acts as a microfilament disrupting agent. The structure of 1 was elucidated by application of spectroscopic methods. The absolute configuration was determined by the exciton chirality method.

As part of a research program aimed at the discovery of new natural products, we have been screening fermentation broths of endophytic fungi from plants growing in Hawaii for activity against the cytoskeleton, specifically, microtubules and microfilaments. Ethyl acetate extracts of broths were examined for anticytoskeletal activity by means of immunofluorescence microscopy procedures.[1]

The crude extracts of strain 112/13,[2] obtained from the bark of *Ficus microcarpa* L., showed strong antimicrofilament activity as evidenced by 50−75% actin filament loss at 5 μg/mL in A-10 cells (rat smooth muscle cells). Hence this isolate was selected for large-scale fermentation (4 L) and isolation of the active principle. Cultivation was performed in 2-L Erlenmeyer flasks containing 0.5 L of potato-dextrose broth for 3 weeks as standing cultures in the dark.

Microcarpalide **1** was isolated by bioassay-guided fractionation after repeated normal-phase chromatography of the ethyl acetate extract (0.25 g) as an optically active, colorless oil (18 mg, 7.2%){$[\alpha]_D$ −22 (*c* 0.67, MeOH)}. The IR spectrum of **1** displayed significant bands at 3380 and 1715 cm^{-1} indicating the presence of alcohol and ester functional groups, respectively. The UV/vis spectrum recorded in MeOH showed end absorption only. Examination of the ^1H NMR spectrum of **1** recorded in CD_3CN indicated the presence of a minor component (ratio of 3.5:1) in the chromatographically apparently homogeneous material. This ratio was a function of the solvent used for NMR, which suggested the presence of conformers. Inspection of the ^{13}C NMR spectrum of **1**, which displayed many doubled and several extensively broadened resonances, corroborated this interpretation.[3] This problem was so severe that even a simple carbon count for **1** could not be obtained with confidence. Further difficulties arose because **1** did not yield a clear-cut

[†] Present address: Southwest Foundation for Biomedical Research, P.O. Box 760549, San Antonio, TX 78245-0549.

(1) For experimental procedure, see: Luesch, H.; Yoshida, W. Y.; Moore, R. E.; Paul, V. J.; Mooberry, S. L. *J. Nat. Prod.* **2000**, *63*, 611−615.

(2) Taxonomic identification of strain 112/13 has so far been unsuccessful. The strain has been deposited in the UH Chemistry Department's culture collection under the accession number 112/13.

(3) Owing to limited solubility of **1** in other common NMR solvents, acetone-d_6, methanol-d_4, DMSO-d_6, and $CDCl_3$ were the only other solvents tried, with inferior results.

10.1021/ol016515s CCC: $20.00 © 2001 American Chemical Society
Published on Web 10/02/2001

molecular ion under a variety of ionization conditions (EI, FAB, CI, ES).

Eventually, a partial gross structure of **1** was established on the basis of the resonances for the major conformer by analysis of COSY, gHSQC, and gHMBC spectra recorded in CD_3CN (see Supporting Information). This analysis suggested that **1** contained a double bond of *trans* geometry ($^3J = 15.8$ Hz) and a vicinal diol function. On the basis of this information, we prepared the ketal derivative **2**, which displayed a much-improved ratio of minor to major conformer of 1:7 in $CDCl_3$ solution (see Supporting Information). Gratifyingly, **2** proved to be amenable to mass spectrometric analysis. The low-resolution EIMS of **2** indicated a molecular mass of 340 and the HREIMS (found 340.2279, $\Delta = -2.9$ mmu) suggested a molecular formula of $C_{19}H_{32}O_5$ for **2** and hence one of $C_{16}H_{28}O_5$ for **1**. The detailed analysis of the COSY, gHSQC, and gHMBC spectra of both **1** and **2** resulted in the assignment of the structure shown in Figure 1. The connectivity within the nonenolide

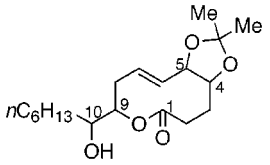

Figure 1. Gross structure of **2**.

ring was deduced from COSY data and is in accord with the gHMBC spectra.

The lactone structure followed from chemical shift considerations for the C-9 hydroxymethine proton and from a gHMBC correlation of H-9 to C-1, which could be observed in the data set of **1**(major) but not in that of **2**.[4] The presence of an exocyclic secondary free hydroxyl group at C-10 was readily inferred from the gHMBC data of **1**.

The structural features present within the ring including C-10 accounted for ten carbon atoms, all of the five oxygen atoms, and fifteen hydrogen atoms of the molecular formula as well as the three degrees of unsaturation of **1**. This in turn suggested the presence of a C_6H_{13} substituent, which had to be placed on C-10. A broad methylene envelope at 1.28−1.40 ppm and a broad three-proton triplet at 0.87 ppm in the ^{1}H NMR spectra of **1** and of **2** suggested the presence of an *n*-hexyl unit.

With the gross structure in hand, we turned to the determination of the relative stereochemistry of the C-4/C-5 ketal function of **2**. The large coupling constant of 8.8 Hz observed between H-4 and H-5 in **2** and the lack of any NOE correlation between these protons was indicative of a *trans* ring fusion and hence of *syn* stereochemistry for the *seco* acid at C-4/C-5.

The relative stereochemistry of the vicinal diol moiety at C-9/C-10 was assigned by application of the method of Murata.[5] A sample of **1** in methanol-d_4 yielded ^{1}H NMR data that suggested the presence of three conformers, the major one amounting to about 70%. The characteristic coupling constants extracted from HETLOC spectra[6,7] of this sample are shown in Table 1. This analysis strongly

Table 1. Characteristic $^{2,3}J$ Values of **1** for the C-9/C-10 Diol Function Measured in Methanol-d_4

	J (Hz) (classification)[b,c]	classification[a] for *threo* diol
^3J (H-9, H-10)	+4.4 (medium)[b,c]	medium
^3J (H-9, C-11)	+1.8 (small)[b]	small
^3J (H-10, C-8)	+1.5 (small)[b]	small
^2J (H-10, C-9)	−0.9 (medium)[b,c]	medium
^2J (H-9, C-10)	−0.3 (medium)[b,c]	medium

[a] According to Figure 5 in ref 5. [b] According to Table 1 in ref 5. [c] Values between *large* and *small* as defined in Table 1, ref 5 are regarded as *medium* (ref 5).

suggested *threo* configuration of the C-9/C-10 diol function of **1**.

Next we established the preferred conformation of **2** with the aid of a ROESY experiment. The most important correlations are shown in Figure 2. Using the H-4/H-5

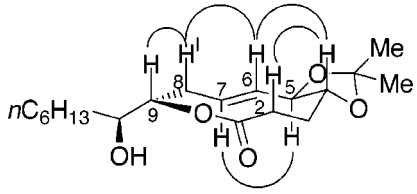

Figure 2. Key ROESY correlations of **2**.

protons of known *anti* orientation as a starting point, the analysis suggests that H-5 and H-7 are placed on one face of the ring, whereas H-4, H-6, H-8^l and H-9 reside on the opposite face.[8]

This NOE-based result was corroborated by coupling constant analysis for **2**, which was of particular importance with respect to the stereochemistry at C-9. Proton H-7

(4) A detailed inspection of the ^{1}H NMR spectrum of **2** suggested that the data for the predominant conformer of **2** are similar to those of the minor conformer of **1**. Thus, $^3J_{H-5/H-6}$ is large (9.4 Hz) in **1**(minor) and in **2**, whereas in **1**(major) $^3J_{H-5/H-6}$ is small (2.0 Hz). Moreover, H-6 resonates upfield of H-7 in **1**(minor) ($\Delta\delta = -0.61$ ppm) and in **2** ($\Delta\delta = -0.45$ ppm) but downfield of H-7 in **1**(major) ($\Delta\delta = +0.2$ ppm). The changes are largely due to the shifting of the resonance of H-6 rather than that of H-7, possibly as a result of reorientation of the diamagnetic shielding cone associated with the lactone carbonyl across the ring.

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(8) H-8^h and H-8^l indicate the H-8 proton resonating at high and low field, respectively.

showed a large coupling to H-8^{l} ($^{3}J_{H-7/H-8} = 11.2$ Hz), which is indicative of a *trans* diaxial orientation. In turn, H-8^{l} coupled to H-9 with $^{3}J_{H-8/H-9} = 8.7$ Hz. The large H-8^{l}/H-9 coupling constant was of some concern as it might suggest an *anti* orientation and hence a *trans* relationship between these two protons rather than a *cis* one, as we had concluded from the ROESY data. If H-8^{l} and H-9 were to reside on the same face of the ring at all, the magnitude of the coupling constant would suggest that they cannot be present in a staggered (*gauche*) conformation in **2** and hence a *syn-periplanar* arrangement would have to be postulated instead. Coupling constant calculations[9] based on dihedral angles of 0° and 180° predicted a value for $^{3}J_{H-8/H-9}$ of 9.4 and 11.4 Hz, respectively. The former value was in excellent agreement with the experimental value of 8.7 Hz and suggested a small dihedral angle between H-8^{l} and H-9. This confirmed our earlier assignment, based on ROESY, of a *cis* relationship between these two protons.

After having secured the H-8^{l}/H-9 stereochemistry, we were now in a position to establish the absolute stereochemistry of the C-9/C-10 *syn* diol function on the basis of the absolute configuration at C-4/C-5.

The latter was readily determined by application of the exciton chirality method.[10] The bis-*p*-methoxy benzoate **3** was prepared by standard methods after acetylation of ketal **2** followed by mild acid hydrolysis. The ^{1}H NMR spectrum of **3**, acquired in CD_3CN at 50 °C, suggested the presence of one major conformer (≥90%). The coupling constant between H-4 and H-5 was determined to be 5.8 Hz. This value is considerably smaller than that observed in **2** for these two protons ($^{3}J_{H-4/H-5} = 8.8$ Hz) and suggested a difference in conformation between **2** and **3**. However, after inspection of Dreiding molecular models this proved to be of no serious concern. The flexibility of the 1-oxa-cyclodecene system present within **3** is not sufficient to allow for inversion of the sense of helicity of the C-4/C-5 dibenzoate function during conformational twisting of the ring at ordinary temperature.

The CD spectrum of **3** in acetonitrile solution showed a first negative and a second positive Cotton effect (λ_{min} 264 nm $\Delta\epsilon = -7.6$, λ_{max}, 246 nm $\Delta\epsilon = +6.5$, $\Sigma\Delta\epsilon = -14.1$). This is indicative of negative exciton chirality and hence of 4*R*,5*R* absolute configuration.[10] Since H-9 resides on the same face of the molecule as H-4, as shown in Figure 2, it follows that the absolute configuration at the C-9/C-10 *threo* diol is 9*S*,10*S*.

Microcarpalide **1** is related to achaetolide,[11] with which it shares the molecular formula, although obvious differences exist with respect to hydroxylation pattern and double bond position. It is also related to a series of 12-carbon polyhydroxy-lactone phytotoxins such as pinolidoxin,[12] lethaloxin,[13] putaminoxin,[14] and the herbarumins,[15] all of which bear a propyl substituent at C-9.

Microcarpalide **1** is but the second C_{16} nonenolide natural toxin described to date and the first one for which a total structure and a molecular mechanism of action, namely disruption of actin filaments, may be proposed. It is tempting to speculate that the propyl-substituted nonenolides share a common mode of action with **1**.

In A-10 cells, pure samples of **1** disrupted microfilaments in approximately half of the cells at a concentration of 0.5−1.0 μg/mL as shown by the immunofluorescence procedure. At concentrations above 20 μg/mL a cytotoxic effect was observed in the A-10 cell line as evidenced by cell loss. The cytotoxicity of **1** against cancer cells is similarly weak with IC_{50} values in the KB (human nasopharyngeal carcinoma) and LoVo (human colon adenocarcinoma) cell lines of 50 and 90 μg/mL, respectively, as determined by the sulforhodamine B assay.[16] The large differential between the antimicrofilament activity and the cytotoxicity potentially makes **1** a tool for studies of cell motility and metastasis.

The most well-known compounds of fungal origin with antimicrofilament activity are the cytochalasins, some of which are several orders of magnitude more potent as cytotoxins than **1**.

Acknowledgment. The authors thank A. Hernandez, G. Tien, and M. Vierra for bioassays and the Victoria L. and Bradley S. Geist Foundation for support of this work. Funds for an upgrade of the NMR instrumentation were provided by the NSF (CHE 9974921) and the Pardee Foundation.

Supporting Information Available: ^{1}H and ^{13}C NMR spectra of **1** and of **2**, tables of NMR data of **1** and of **2**, and CD spectrum of **3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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