Mollenines A and B: New Dioxomorpholines from the Ascostromata of *Eupenicillium molle*

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Two new dioxomorpholines (**1** and **2**) have been isolated from the sclerotioid ascostromata of *Eupenicillium molle* (NRRL 13062). Their structures were determined by analysis of NMR data. Mollenine A (**1**) exhibited moderate cytotoxicity and antibacterial activity, but neither compound displayed significant antiinsectan activity.

Our interest in fungal sclerotia and similar physiological structures as sources of new antiinsectan metabolites[1,2] prompted us to investigate the chemistry of the sclerotioid ascostromata of *Eupenicillium* spp. Ascostromata are specialized fungal bodies, analogous to sclerotia, that are adapted to withstand extreme conditions of temperature, desiccation, and nutrient depletion. Our studies of ascostromata produced by *E. shearii* and *E. crustaceum* have afforded a number of metabolites with activity against the corn earworm *Helicoverpa zea*.[2−4] As a part of this project, we undertook an exploration of the chemistry of the ascostromata of *Eupenicillium molle* Malloch et Cain (Trichocomaceae) (NRRL 13062). Preliminary assays indicated that the hexane extract exhibited significant activity against both *H. zea* and the fungivorous beetle *Carpophilus hemipterus* in dietary assays. During the investigation of this extract, two new metabolites were encountered, which we have named mollenines A (**1**) and B (**2**). This report describes the isolation, structure elucidation, and bioassay results for these compounds.

Ascostromata of *E. molle* (NRRL 13062) were produced by solid substrate fermentation on corn kernels.[4] The antiinsectan hexane extract of the ascostromata was fractionated on a Sephadex LH-20 column, and active fractions were further separated by preparative reversed-phase HPLC to afford mollenines A (**1**) and B (**2**).

HREIMS data established the molecular formula for mollenine A (**1**) as $C_{22}H_{28}N_2O_3$. Signals accounting for all 28 protons (only one exchangeable) and 22 carbons were observed in the ^{1}H, ^{13}C, and DEPT NMR spectra (Table 1). Four structural units, consisting of an *ortho*-disubstituted benzenoid moiety, a prenyl group, an isolated CH_2-CH-N unit, and partial structure **A** (Figure 1) were recognized upon analysis of ^{1}H and ^{13}C NMR data, and later confirmed by DEPT, COSY, HMQC, and HMBC results.

The presence of an NH group was suggested by a weak IR absorption at 3411 cm^{-1} and an exchangeable proton NMR signal at δ 5.09. This NH group was

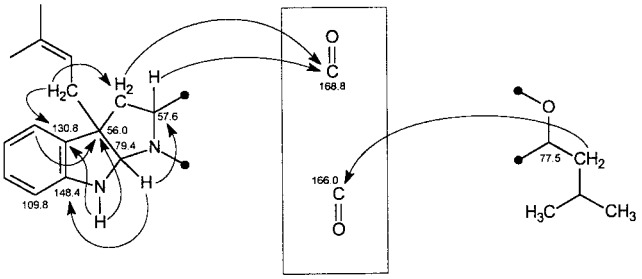

Figure 1. Partial structures and units with selected HMBC correlations.

connected to the benzene ring carbon at δ 148.4, based on the chemical shift of this signal and an HMBC correlation from the NH proton to the adjacent aromatic carbon at δ 130.8 (C-13; Figure 1). The presence of one amide and one ester group was indicated by the carboxyl carbon signals at δ 166.0 and 168.8, together with IR absorptions at 1679 and 1761 cm^{-1}. These units account for all of the heteroatoms in the formula, except for one nitrogen atom, and require that the oxygen atom linked to the methine carbon in partial structure **A** must be acylated by the ester carbonyl. The presence of an additional downfield-shifted methine carbon signal at δ 79.4 (C-6) could only be accounted for by its connection to both nitrogen atoms. The corresponding isolated methine proton (δ 5.26) showed an HMBC correlation to the NH-substituted aromatic carbon (δ 148.4), leading to attachment of this methine group (position 6) to the NH and requiring that the other nitrogen atom be part of the amide unit. Additional HMBC correlations for H-6, NH-7, H-12, H_2-15, and H_2-17 (see Table 1) linked C-6 and the prenyl unit to the quaternary carbon at δ 56.0 (C-14) and permitted the connection of C-14 to the aromatic carbon at δ 130.8 (C-13) to form a five-membered ring. HMBC correlations of the H_2-17 protons of the prenyl group to the methylene carbon in the CH_2-CH-N unit (C-15) required linkage of C-15 with C-14 to form a second five-membered ring. These data led to the assignment of partial structure **B** (Figure 1).

Partial structures **A** and **B**, together with the two carbonyls, accounted for all of the atoms of mollenine A. The only remaining element was one degree of

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Table 1. NMR Data for Mollenines A (**1**) and B (**2**) ($CDCl_3$)

position	**1** δ ^{1}H (mult., J in Hz)[a]	**1** ^{13}C (δ)[b]	**2** δ ^{1}H (mult., J in Hz)[a]	**2** ^{13}C (δ)[b]	HMBC correlations for **1** (C#)
1		168.8		168.0	
3	4.68 (dd, 10, 2.8)	77.5	4.74 (dd, 9.9, 2.8)	77.3	22, 23
4		166.0		165.5	
6	5.26 (s)	79.4	5.79 (s)	78.4	8, 13, 16, 17
7 (NH)	5.09 (s)				13, 14
8		148.4		140.1	
9	6.60 (dd, 8.0, 0.8)	109.8	8.01 (d, 7.9)	117.3	10, 11, 13
10	7.08 (ddd, 8.0, 7.5, 1.2)	123.2	7.30 (dd, 7.9, 7.5)	129.4	8, 12
11	6.77 (ddd, 7.5, 7.5, 0.8)	119.7	7.15 (dd, 7.6, 7.5)	125.5	9, 10, 12, 13
12	7.08 (dd, 7.5, 1.2)	128.9	7.23 (d, 7.6)	123.1	8, 10, 14
13		130.8		133.9	
14		56.0		54.9	
15	2.68 (dd, 13, 6.7)	37.7	2.79 (dd, 13, 6.4)	38.8	6, 13, 14, 16
	2.41 (dd, 13, 11)		2.43 (m)		1, 13, 14, 16, 17
16	4.10 (dd, 11, 6.7)	57.6	4.16 (dd, 11, 6.4)	57.4	1, 15
17	2.38 (dd, 14, 7.6)	35.0	2.43 (m)	35.8	6, 13, 14, 15, 18, 19
	2.32 (dd, 14, 7.6)				6, 13, 14, 15, 18, 19
18	5.13 (dd, 7.6, 7.6)	118.0	5.01 (dd, 7.0, 7.0)	116.9	17, 20, 21
19		136.2		137.5	
20	1.51 (br s)	17.9	1.52 (s)	18.0	18, 19, 21
21	1.69 (br s)	26.0	1.68 (s)	25.9	18, 19, 20
22	1.93 (m)	37.7	1.93 (m)	37.7	3, 23, 24, 25
	1.78 (ddd, 10, 10, 3.0)		1.79 (dd, 10, 10)		3, 4, 23, 24
23	1.91 (m)	24.0	1.93 (m)	23.9	3, 24, 25
24	0.89 (d, 6.4)	21.2	0.90 (d, 6.3)	21.2	22, 23, 25
25	0.95 (d, 6.4)	23.2	0.96 (d, 6.3)	23.2	22, 23, 24
26			9.01 (s)	161.7	

[a] 600 MHz. [b] 75 MHz. Multiplicities for the carbon resonances were deduced from DEPT experiments and are consistent with the above assignments.

unsaturation, which must be accounted for by a ring. The only logical assembly of the two partial structures leads to formation of a dioxomorpholine ring, and this conclusion was supported by observation of additional HMBC correlations to the carbonyl carbons as shown in Figure 1. Based on these data, the gross structure of mollenine A was established as **1**.

The relative stereochemistry of **1** was deduced from NOESY data. The signal for H-16 correlated to signals for H-3 and H-6, placing these three protons on the same face of the molecule and indicating that the dioxomorpholine ring adopts a boatlike conformation. In addition, correlations of the H-17, -18, -20, and -21 signals with H-6 indicated a cis relationship between H-6 and the prenyl group. To determine the absolute configuration of mollenine A, a sample of **1** was treated with 6N HCl under standard hydrolysis conditions. The resulting mixture was extracted with Et_2O, and evaporation of the solvent afforded 2-hydroxyisocaproic acid. The specific rotation of this product was found to be opposite to that of an authentic standard of (−)-*S*-2-hydroxyisocaproic acid, and the reaction product was therefore assigned the *R* configuration. Thus, the absolute stereochemistry proposed for mollenine A is shown in structure **1**. Analysis of the aqueous residue by NMR failed to detect the presence of tryptophan and indicated that the prenyl side chain remained on the tryptophan-derived subunit, even after prolonged exposure to hydrolysis conditions.

The occurrence of the 16*R* configuration in **1** was initially surprising, because the *S* configuration would be predicted based on the presumed intermediacy of tryptophan. There are only a few examples of tryptophan-derived fungal metabolites having the *R* configuration at an analogous position.[5] However, two NMR experiments clearly demonstrated that H-16 is labile. When Na_2CO_3 was added to a solution of **1** in CD_3OD, the H-16 signal disappeared, and the signals for H_2-15 simplified to doublets. The same result occurred when a separate sample of **1** in CD_3OD was merely warmed to 55−60 °C. Evaporation of the latter sample, followed by treatment with MeOH, led to reprotonation and restoration of these signals in the NMR spectrum ($CDCl_3$). The signal for H-3 did not change in either experiment, indicating that only H-16 is susceptible to exchange under these conditions. Thus, it is proposed that mollenine A is derived from the condensation of a modified L-tryptophan residue with an *R*-2-hydroxyisocaproic acid moiety, and that the unusual stereochemistry at C-16 arises from epimerization at that center. Presumably, the increased lability of H-16 in **1** relative to otherwise similar dioxopiperazine-type compounds[6−8] is due to the fact that this proton is α to an ester rather than to an amide. The predominance of **1** suggests that it is thermodynamically favored over the 16*S* diastereomer.

A ^{1}H NMR spectrum ($CDCl_3$ solution) of the crude hexane extract from which mollenine A was isolated clearly contained a signal for H-16 with the same chemical shift and J values observed in the spectrum of **1**. This spectrum was recorded before the extract was exposed to MeOH or to chromatographic conditions, suggesting that epimerization occurred before isolation, possibly even during the fermentation process.

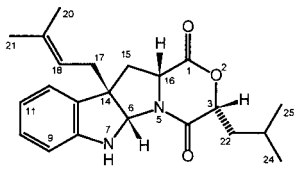
Interestingly, a peak corresponding to an (M + H + solvent) ion was observed in the FABMS of mollenine A when the sample was obtained by evaporation from MeOH (or EtOH). Upon evaporation of a sample from MeOH and subsequent NMR analysis in $CDCl_3$, two additional sets of minor signals were observed in addition to those expected for **1**. Although neither of these minor components was formed in sufficient quantities

for isolation, it was possible to identify them as ring-opened 16*R*- and 16*S*-epimeric methyl esters by analysis of 1D NMR, HMQC, and HMBC data ($CDCl_3$) obtained for the mixture. A characteristic pair of methoxy singlets (1.2:1 ratio) was observed at δ 3.72 and 3.79, corresponding to the methyl ester groups. As expected, the signals for H-3 in these ring-opened products resonate upfield from those of the closed-ring form (δ 4.35 and 3.84, as opposed to δ 4.67). These results are apparently due to partial conversion of the dioxomorpholine ring structure to a ring-opened form (i.e., lactone solvolysis product) in MeOH, accompanied by epimerization at C-16. The ring-opening process is consistent with that observed for δ lactones. For example, mevalonic acid undergoes an analogous ring-opening in protic solvents.[9] Despite the appearance of both epimers of these open-chain products, only one closed-ring epimer of mollenine A was observed in solution, even after exposure to epimerization conditions. Thus, as might be predicted, there appears to be a greater energy difference between the two possible epimers in the more conformationally restricted closed-ring form.

The EIMS of mollenine B (**2**) contained a molecular ion 28 mass units larger than that of **1**, though the fragmentation patterns were otherwise very similar. HRFABMS data indicated the presence of an additional CO unit, resulting in a molecular formula of $C_{23}H_{28}N_2O_4$ for **2**. The ^{1}H NMR spectrum was nearly identical to that of mollenine A, except that it lacked the NH signal and contained instead a new singlet at δ 9.01. Based on HMQC data, this proton is directly connected to a carbon with a chemical shift of δ 161.7, indicating the presence of a formyl group. An HMBC correlation of the formyl proton with C-8 revealed that the formyl group replaced the amine hydrogen atom in **1**. Therefore, the structure of mollenine B was assigned as **2**. Based on their similar NMR characteristics, the relative and absolute stereochemistry for mollenine B is presumed to be analogous to that of mollenine A.

Mollenines A and B did not show activity in assays against *H. zea* larvae[10] at the 100 ppm dietary level and are not responsible for the antiinsectan activity of the original extract. Mollenine A however, did display antibacterial activity in a standard disk assay against *Bacillus subtilis* at 200 μg/disk and displayed moderate cytotoxicity in the NCI's 60-tumor cell line panel,[11] with an average GI_{50} value of 3.1×10^{-5} M (11.4 μg/mL).

Mollenines A and B (**1** and **2**) are novel metabolites of mixed biogenesis, containing amino acid, α-hydroxy acid, and isoprene units. These metabolites differ from known amino acid-derived dioxopiperazine structures[7] in that they apparently result from condensation of an amino acid subunit with an α-hydroxy acid moiety. The closest known dioxopiperazine analogue to **1** and **2** is verrucofortine (**3**), also known as fructigenine B, which has been isolated from *Penicillium verrucosum* and *P. fructigenum*.[8,9] The absolute stereochemistry reported for **3** was determined after comparison of a dioxopiperazine degradation product (obtained by mild acid hydrolysis of **3**) with an authentic standard prepared from L-tryptophan and L-leucine.[8] Interestingly, the absolute stereochemistry assigned at C-3 and C-16 for mollenines A and B is opposite to that of **3**. Although dioxopiperazines are common as fungal metabolites, dioxomorpholines are quite rare. In fact, considering the presence of the ester oxygen atom, the ring system found in mollenines A and B has not been reported previously.



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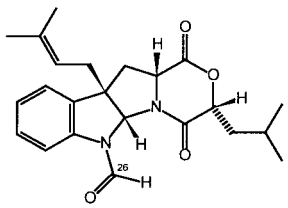

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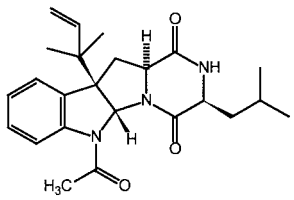

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Experimental Section

General Experimental Procedures. ^{1}H and ^{13}C NMR spectra were obtained on a Bruker AC-300 instrument. HMQC and HMBC experiments were performed on a Bruker AMX-600 spectrometer and were optimized for $^1J_{CH} = 152$ Hz and $^nJ_{CH} = 8$ Hz, respectively. All NMR spectra were recorded using $CDCl_3$ solutions, unless otherwise noted. Chemical shift values were referenced to the corresponding solvent signal(s). FABMS data were recorded on a VG ZAB−HF mass spectrometer.

Organism and Culture. The culture of *E. molle* NRRL 13062 (ex type culture; = CBS 456.72) was obtained from the Agricultural Research Service Collection at the National Center for Agricultural Utilization Research in Peoria, IL. The culture was originally isolated by S. A. Lodhi from soil collected in Pakistan. Ascostromata of *E. molle* were produced by solid substrate fermentation on autoclaved corn kernels and were harvested using procedures previously described.[4]

Isolation of Mollenines A and B. A sample of intact, dry ascostromata of *E. molle* (NRRL 13062; 135 g) was extracted at 25 °C with hexane. After removal of solvent, 4.7 g of an antiinsectan crude extract was obtained. A portion of the extract (1.08 g) was fractionated on a Sephadex LH-20 column (2.8 × 43 cm), eluting with CH_2Cl_2−MeOH (1:1). Similar fractions were combined based on TLC analysis. Fractions 6 and 7 (125 of 156 mg) were processed by preparative reversed-phase HPLC (80% CH_3CN-H_2O) to yield mollenine A (**1**, 8.4 mg, t_R 18.5 min) and mollenine B (**2**, 1.2 mg, t_R 14.5 min).

Mollenine A (1): colorless oil from CH_2Cl_2; $[\alpha]_D$ −410° (*c* 1.0 mg/mL, CH_2Cl_2); UV (CH_2Cl_2) λ_{max} 240 (4900), 295 (1700); EIMS (70 eV) *m/z* 368 ($M^{\bullet+}$; rel int. 24%), 299 (63), 157 (69), 130 (100), 69 (16); IR 3411, 2962, 1761, 1679 cm^{-1}; ^{1}H NMR, ^{13}C NMR, and HMBC data, see Table 1; HREIMS, $M^{\bullet+}$ at *m/z* 368.2119, calcd for $C_{22}H_{28}N_2O_3$, 368.2100.

Mollenine B (2): colorless oil from CH_2Cl_2; $[\alpha]_D$ −180° (*c* 1.0 mg/mL, CH_2Cl_2); UV (CH_2Cl_2) λ_{max} 251 (ϵ

9000), 282 (2000); EIMS (70 eV) *m/z* 396 ($M^{\bullet+}$; rel int. 2%), 368 (17), 299 (45), 157 (55), 130 (100), 69 (29); IR 3061, 2963, 1762, 1692 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; HRFABMS (thioglycerol) $(M + H)^+$ at *m/z* 397.2116, calcd for $C_{23}H_{28}N_2O_4$+H, 397.2127.

Hydrolysis of mollenine A (1): 6N HCl (1 mL) was added to 14 mg of mollenine A in a hydrolysis tube. The solution was allowed to stand at 110 °C for 24 h, then cooled to room temperature and extracted with Et_2O (5 mL × 3). The combined Et_2O fractions were evaporated to afford 3.1 mg (62% yield) of 2-hydroxyisocaproic acid, as identified by ^1H NMR data (recorded in CD_3OD solution) and EIMS results. The specific rotations of this product and an authentic standard of (−)-*S*-hydroxyisocaproic acid were found to have $[\alpha]_D$ values equal in magnitude, but opposite in sign.

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Supporting Information Available: ^1H NMR, ^{13}C NMR, and DEPT spectra for mollenine A (**1**) (2 pages). Ordering information is given on any current masthead page.

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