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Thiersinines A and B: Novel Antiinsectan Indole Diterpenoids from a New Fungicolous *Penicillium* Species (NRRL 28147)

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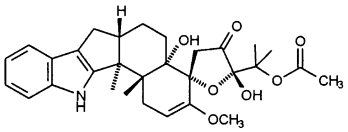
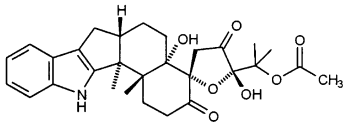
ABSTRACT



Thiersinine A

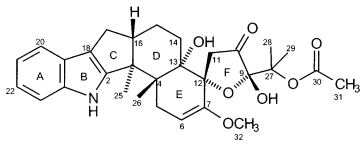


Thiersinine B

Two novel antiinsectan indole diterpenoids, thiersinines A (1) and B (2), along with seven known paxilline-type compounds, were isolated from organic extracts of a new *Penicillium* species (*P. thiersii* NRRL 28147). The structures of 1 and 2 were determined by analysis of 2D NMR data. Thiersinines A and B possess a unique spirocyclic subunit that is unprecedented in previously known compounds of this class. Both compounds exhibit potent activity against the fall armyworm (*Spodoptera frugiperda*).

Our studies of *Aspergillus*, *Eupenicillium*, and *Penicillium* spp. have led to the discovery of many new antiinsectan compounds,[1−3] despite extensive prior chemical investigations of these genera. Our prior work with these taxa involved studies of extracts from sclerotia or ascostromata produced by these fungi. However, our recent focus on investigations of fungicolous and mycoparasitic fungi that colonize sclerotia or stromata of wood decay fungi[4,5] have led to the isolation of a variety of distinctive isolates of *Penicillium*, many of which are proving to be new species. Chemical investigation of one such new species, *Penicillium thiersii* (MYC-500 = NRRL 28147), afforded two novel antiinsectan indole diterpenoids that we named thiersinine A (**1**) and B (**2**), along with seven known indole diterpenoids. Details of the isolation, structure determination, and biological activities of thiersinines A and B are presented here.

The EtOAc extract of solid-substrate fermentation cultures of *P. thiersii* exhibited potent antiinsectan activity against the agriculturally important fall armyworm (*Spodoptera frugiperda*). Bioassay-guided fractionation of this extract



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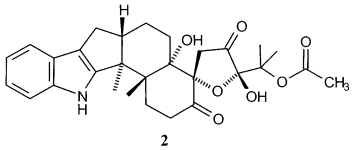

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afforded two new indole diterpenoids (thiersinines A and B; **1** and **2**),[6] as well as paxilline,[7] paspaline,[8] dehydroxypaxilline,[9] emindole SB,[9] 1′-*O*-acetylpaxilline,[10] PC-M5′, and PC-M6.[11] The structures of the known compounds were confirmed by comparison of their NMR and MS data with literature values.

The molecular formula of thiersinine A (**1**) was determined to be $C_{30}H_{37}NO_7$ (13 unsaturations) by analysis of ^{1}H, ^{13}C, and DEPT NMR data and was verified by HRESIMS. The EI mass spectrum contained a base peak at *m*/*z* 182 characteristic of the 2,3-disubstituted indole moiety commonly found in the paspaline/paxilline class, and the five downfield signals in the aromatic region of the ^{1}H NMR spectrum also indicated the presence of a 2,3-disubstituted indole unit. Comparison of the ^{1}H and ^{13}C NMR data for compound **1** with those of the seven known indole diterpenoids listed above strongly suggested the existence of the same A−D structural ring unit in all nine compounds. This assignment was confirmed by detailed analysis of the COSY, HMQC, and HMBC data for **1** (Table 1).

By contrast, the data for the remaining portion of the structure were quite distinctive from those of known members of the paxilline class. These data included signals for a ketone, a methoxy group, a ketal or hemiketal carbon, a pair of geminal methyl groups, two oxygenated quaternary sp^3 carbons, an isolated methylene unit, an acetate group, and an oxygenated olefin unit. The elemental composition of **1** also required the incorporation of a second OH group

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

position	δ_H (mult; *J*, Hz)	δ_C	HMBC (H# → C#)
1 NH	10.68 (s)		2, 18, 19, 24
2		152.9	
3		51.2	
4		42.5	
5a	2.18 (dd; 6, 16)	29.5	4, 6, 7, 13, 26
5b	2.88 (br d; 16)		3, 4, 6, 7, 26
6	4.88 (br dd; 1.2, 6.0)	95.7	4, 5, 7, 12
7		153.0	
9		98.4	
10		211.3	
11a	2.42 (d; 18)	45.0	7, 9, 10, 12, 13
11b	3.14 (d; 18)		7, 10, 12, 13
12		82.0	
13		78.5	
14a	1.94 (m)	30.0	4, 13, 15, 16
14b	2.03 (br dd; 4, 13)		15
15a	1.62 (m)	21.0	3, 13, 14
15b	1.92 (m)		3, 13, 16
16	2.67 (m)	49.1	3, 15, 17, 25
17a	2.30 (dd; 11, 13)	26.9	2, 15, 16, 18
17b	2.59 (dd; 6, 13)		2, 3, 16, 18
18		114.8	
19		124.5	
20	7.26 (dd; 1.2, 7.8)	117.7	18
21	6.89 (br dd; 7.8, 8.4)	118.4	19, 23
22	6.93 (br dd; 7.8, 8.4)	119.3	20, 23
23	7.26 (dd; 1.2, 7.8)	111.7	19, 21, 24
24		140.1	
25	1.30 (s)	16.6	2, 3, 4, 16
26	0.99 (s)	20.4	3, 4, 5, 13
27		84.8	
28	1.55 (s)	19.7	9, 27, 29
29	1.61 (s)	19.5	9, 27, 28
30		168.9	
31	1.93 (s)	22.1	27,[b] 30
32	3.55 (s)	55.1	7
9-OH	4.95 (br s)		9, 10, 27
13-OH	3.74 (br s)		4, 12, 13

[a] Data were recorded in DMSO-d_6 at 600 and 90.7 MHz, respectively. The numbering system was chosen to permit facile comparison with other members of the paxilline/paspaline class. [b] A four-bond correlation.

(6) The culture employed in this work (MYC-500 = NRRL 28147) was determined by Dr. S. W. Peterson of the USDA National Center for Agricultural Utilization Research (NCAUR) in Peoria, IL to be a previously undescribed species, and assigned the name *P. thiersii*.[22] A subculture has been deposited in the Agricultural Research Service (ARS) collection at the NCAUR. The strain was originally isolated from an old stroma of *Hypoxylon* sp. found at New Glaurus Woods State Park, near New Glaurus, WI, that was collected by Dr. H. D. Thiers on August 21, 1996. General fermentation procedures used have been published elsewhere.[5] The EtOAc extract (3.6 g) from rice solid-substrate fermentation cultures (carried out in eight 500 mL Erlenmeyer flasks, each containing 50 g of rice) of *P. thiersii* was partitioned between CH_3CN and hexane. The CH_3CN-soluble portion (2.3 g) was subjected to Sephadex LH-20 column chromatography using a hexane−CH_2Cl_2−acetone solvent gradient, and the third fraction eluted with 1: 4 hexane−CH_2Cl_2 (71 mg) was further separated by reversed-phase HPLC (Alltech HS Hyperprep 100 BDS C_{18} column, 5-μm particles, 1.0 × 25 cm; flow rate 2.0 mL/min; 60% to 100% CH_3CN in H_2O over 40 min) to yield thiersinine A (**1**, 20 mg, t_R 27.2 min). Compound **1** has the following properties: white solid; mp 255−258 °C; $[\alpha]_D$ − 80 (*c* 0.3, CH_2Cl_2); UV (MeOH) λ_{max} 230 (ϵ 17 000), 272 (ϵ 3000); IR (CH_2Cl_2) ν_{max} 3434, 2938, 1745, 1718, 1676, 1472, 1372, 1258, 1230, 1162, 1025 cm^{-1}; ^{1}H NMR, ^{13}C NMR, and HMBC data, see Table 1; EIMS (70 eV) *m*/*z* 523 (M^+; rel int 56), 508 (60), 351 (18), 334 (27), 208 (23), 182 (100), 167 (42); HRESIMS obsd *m*/*z* 524.2641, calcd for $C_{30}H_{37}NO_7$ + H, 524.2647. The fifth fraction from the LH-20 column which was also eluted with 1:4 hexane−CH_2Cl_2 (99 mg) was further separated on a silica gel column. A subfraction eluted with 3:2 hexanes−EtOAc (12 mg) was further separated by reversed-phase HPLC (same column conditions as above) to yield thiersinine B (**2**, 1.9 mg, t_R 24.6 min) as a colorless oil: $[\alpha]_D$ −93 (*c* 0.1, CH_2Cl_2); UV (MeOH) λ_{max} 230 (ϵ 20 300), 284 (ϵ 3800); IR (CH_2Cl_2) ν_{max} 3403, 2954, 1770, 1734, 1701, 1456, 1367, 1253 cm^{-1}; ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.68 (1H, s, H-1), 7.44 (1H, dd, 1.8, 6.6, H-20), 7.30 (1H, dd, 1.8, 6.6, H-23), 7.10 (1H, ddd, 1.2, 7.2, 7.2, H-22), 7.08 (1H, ddd, 1.2, 7.2, 7.2, H-21), 5.61 (1H, s, 9-OH), 2.96 (1H, ddd, 6, 12, 12, H-5ax), 2.80 (1H, d, 17, H-11a), 2.80 (1H, m, H-16), 2.77 (2H, m, H_2-6), 2.75 (1H, m, H-17a), 2.66 (1H, s, 13-OH), 2.54 (1H, d, 17, H-11b), 2.44 (dd, 11, 13, H-17b), 2.07 (1H, m, H-15a), 2.04 (1H, m, H-14eq), 1.94 (3H, s, H_3-31), 1.86 (1H, ddd, 5, 13, 13, H-14ax), 1.74 (1H, br d, 14, H-15b), 1.71 (6H, s, H_3-28, H_3-29), 1.64 (1H, m, H-5eq), 1.33 (3H, s, H_3-25), 1.27 (3H, s, H_3-26); ^{13}C NMR (100 MHz, $CDCl_3$) δ 214.1 (C-7), 207.7 (C-10), 168.6 (C-30), 151.1 (C-2), 140.0 (C-24), 125.1 (C-19), 120.9 (C-22), 119.9 (C-21), 118.7 (C-20), 117.9 (C-18), 111.5 (C-23), 98.8 (C-9), 88.9 (C-12), 85.3 (C-27), 82.2 (C-13), 52.6 (C-3), 49.4 (C-16), 44.1 (C-11), 43.0 (C-4), 35.1 (C-6), 29.9 (C-14), 29.0 (C-5), 27.2 (C-17), 22.5 (C-31), 20.9 (C-15), 19.9 (C-28), 19.9 (C-26), 19.3 (C-29), 16.4 (C-25); HMBC data ($CDCl_3$) H-1 → C-2, 18, 19, 24; H-5ax → C-3, 4, 6, 26; H-5eq → C-4, 6, 7, 13, 26; H_2-6 → C-4, 5, 7, 12; H-11a → C-7, 9, 10, 12, 13; H-11b → C-7, 9, 10, 12, 13; H-14ax → C-13, 15, 16; H-14eq → C-4, 13, 16; H-15a → C-3, 13, 14, 16; H-15b → C-3, 13, 14, 16; H-16 → C-3, 14, 15, 17, 25; H-17a → C-2, 3, 16, 18; H-17b → C-2, 15, 16, 18; H-20 → C-18, 19, 21, 22, 24; H-21 → C-19, 22, 23; H-22 → C-21, 24; H-23 → C-19, 21, 24; H_3-25 → C-2, 3, 4, 16; H_3-26 → C-3, 4, 5, 13; H_3-28 → C-9, 27, 29; H_3-29 → C-9, 27, 28; H_3-31 → C-27, 30; 9-OH → C-9, 27; 13-OH → C-4, 12, 13, 26; EIMS (70 ev) *m*/*z* 509 (M^+; rel int 12), 494 (6), 449 (5), 434 (6), 320 (3), 238 (7), 182 (44), 167 (19); HRESIMS obsd *m*/*z* 532.2307, calcd for $C_{29}H_{35}NO_7$ + Na, 532.2311.

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(aside from OH-13) and two additional rings. Establishment of the connectivity of these units was complicated by the limited number of protonated carbons in this portion of the molecule.

The ketone carbonyl of **1** (δ 211.3) was significantly downfield-shifted relative to other members of this class, indicating the absence of the typical α,β-unsaturated ketone unit. The methoxy signal at δ 3.55 (H_3-32) showed an HMBC correlation with oxygenated olefinic carbon C-7 (δ 153.0), locating this group at C-7. This placement is consistent with the upfield shift of the neighboring protonated olefinic carbon (C-6; δ 95.7). The singlet corresponding to H_3-26 showed a correlation to methylene carbon C-5, as well as to C-3, C-4, and C-13 of the D-ring. C-5, in turn, was linked to olefinic carbon C-6 on the basis of COSY correlations between H_2-5 and H-6. A correlation of H-6 with C-12 required attachment of C-7 to C-12. The chemical shifts of H_2-11 and C-11 (δ_H 2.47, 3.14; δ_C 45.0) precluded direct attachment of C-11 to oxygen but did suggest location α to a carbonyl or olefinic unit. Correlations of H_2-11 with C-7, oxygenated quaternary carbon C-12 (δ 82.0), and C-13 of the D-ring required direct connection of C-11 to C-12 and permitted closure of the E-ring by linking C-12 and C-13. The latter connection was further supported by a correlation of the 13-OH proton with C-12.

Correlations of the two geminal methyl signals (H_3-28 and H_3-29) with ketal or hemiketal carbon C-9 (δ 98.4), as well as to the remaining oxygenated quaternary carbon (C-27; δ 84.8), enabled linkage of C-9 to C-27. Further HMBC correlations of H_2-11 with C-9 and the ketone carbon (C-10) required connection of both C-11 and C-9 to C-10. At this point, only the acetate unit, a free OH group, and one final ring remained to be assigned, but several different structures were still possible. HMBC correlations between the 9-OH proton and both C-9 and C-10 narrowed the possibilities to three, which would contain either epoxide, furanone, or pyranone rings, although the epoxide possibility was unlikely on the basis of ^{13}C NMR chemical shift considerations. Unambiguous distinction among the three possibilities was ultimately made possible only by a weak, but distinct, four-bond HMBC correlation observed between H_3-31 and C-27. Such correlations are seldom reported for acetate groups, but have been observed previously by others.[12,13] This located the acetate at C-27, rather than at one of the other quaternary carbon positions, and established the final ring as a furanone resulting from linkage of C-9 and C-12 via an oxygen atom as shown in **1**.

The EI mass spectrum of thiersinine B (**2**) had the same characteristic base peak (*m*/*z* 182) as **1**, but indicated a molecular weight 14 mass units lower. The signals in its ^1H and ^{13}C NMR spectra closely matched those of compound **1**. The only significant differences included the presence of a new ketone carbonyl signal at δ 207.7, the absence of the C6−C7 double bond, and the lack of the methoxy signal (H_3-32). These observations strongly suggested that compound **2** differed from **1** in that the methyl enol ether unit was replaced by a ketone group. Analysis of COSY, HMQC, and HMBC data affirmed this assignment. The key four-bond HMBC correlation between H_3-31 and C-27 observed in **1** was again observed for **2**, verifying the location of the acetate group. Thus, thiersinine A (**1**) is the methyl enol ether of thiersinine B (**2**). This relationship was confirmed by acid hydrolysis of **2** to give **1**.[14,15] It is unlikely that **1** is an artifact arising from **2**. Methanol was not employed in the isolation process, and the methoxy signal (for H_3-32 of **1**) was readily discerned in the ^1H NMR spectrum of the original EtOAc extract of *P. thiersii*. On the other hand, it is possible that at least some of the sample of relatively minor component **2** was obtained as a result of incidental hydrolysis of **1**.

The relative stereochemistry for **1** and **2** was assigned by analysis of NOESY data. Strong correlations between H_3-25 and the C-13 OH proton (δ 3.74) and between H_3-26 and H-16 in **1** suggested their relative orientations as shown. This arrangement matches that found in all other known members of the paxilline class. The configuration at the spiro center was assigned as shown on the basis of correlations observed between H_3-26 and both C-11 protons. Finally, a strong correlation between H_3-32 and the C-9 OH signal allowed assignment of the relative stereochemistry at C-9 as reflected in **1**. The NOESY data for compound **2** were consistent with assignment of the same relative stereochemistry as in **1**. The only configuration that could not be independently assigned in **2** was that of C-9, due to a lack of relevant correlations. The absolute stereochemistry of several paxilline-type compounds has been determined,[7−8,16] and thiersinines A and B presumably possess the analogous absolute stereochemistry, as depicted in **1** and **2**.

To date, over 50 paspaline/paxilline-type indole diterpenes have been isolated from various fungal sources. Among the most important members of this general class are the nodulisporic acids, originally reported by researchers at Merck in 1997 as highly potent antiparasitic and insecticidal agents.[17] Although the thiersinines clearly bear a close biogenetic relationship to other indole diterpenoids from this class, particularly paspalinine,[18,19] they are the first representatives having this ring system to be described. Interestingly, only a few natural products that contain enol methyl ether units lacking a conjugated carbonyl unit have been previously reported. Examples include some of the strobil-

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(14) Compound **1** (0.5 mg) was dissolved in THF (100 μL), to which two drops of 3N aqueous HCl solution was added. The solution was stirred at room temperature under N_2.[15] After 70 h, TLC detection showed no remaining starting material and the solution was evaporated to afford thiersinine B (**2**), as confirmed by ^1H NMR, HPLC, and TLC analysis.

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urins,[20] a group of important antifungal lead compounds from basidiomycetes.

Thiersinines A and B exhibit potent activity[21] against *S. frugiperda* when incorporated into a standard test diet at 100 ppm, causing 83% and 84% reduction in growth rate relative to controls, respectively. They were both inactive against *C. albicans* (ATCC 90029) and *S. aureus* (ATCC 29213) in standard disk assays at 200 μg/disk.

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Supporting Information Available: ^{1}H and ^{13}C NMR spectra for thiersinines A (**1**) and B (**2**). This material is available free of charge via the Internet at http://pubs.acs.org.

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