Immunochemical Techniques in Natural Products Chemistry: Isolation and Structure Determination of a Novel Indole–Diterpenoid Aided by TLC–ELISAgram

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Paxinorol, a novel modified indole–diterpenoid believed to be derived from paxilline, was detected with the recently-developed TLC–ELISAgram technique as a trace contaminant of a paxilline-containing extract from the fungus *Penicillium paxilli* Bainier. Paxinorol was isolated by column chromatography, and its structure was determined by ^1H and ^{13}C NMR spectroscopy and mass spectrometry.

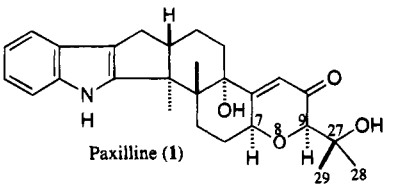
Introduction

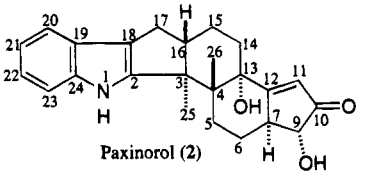
We have developed enzyme-linked immunosorbent assays (ELISAs) for a number of indole–diterpenoids associated with the neurotoxic disease of livestock known as ryegrass staggers, including an ELISA for paxilline.[1] The immunization and selection procedures were such that the anti-paxilline monoclonal antibody used in this immunoassay was capable of recognizing an array of compounds structurally related to paxilline. Recently, Pestka[2] published details of an immunoblot technique coupled to TLC analysis (TLC–ELISAgram). The method, as reported, was essentially a quantitative technique. However, we anticipated that, by using antibodies with broad specificity, TLC–ELISAgram would be an ideal technique for identifying small quantities of structurally-related compounds whose existence in a sample had not previously been suspected.

We now report application of the TLC–ELISAgram technique to the analysis of an extract from *Penicillium paxilli* Bainier, leading to the identification of a trace contaminant. The structure of this contaminant, which we name paxinorol (**2**), was similar to that of the indole–diterpenoid skeleton of paxilline (**1**), but with a highly modified F-ring.

Results and Discussion

TLC of the *P. paxilli* extract indicated that the major component (R_f 0.8) was paxilline. This was confirmed by isolation of this component (200 mg) and comparison of its ^1H and ^{13}C NMR and mass spectra with those reported for paxilline in the literature.[3] Several minor spots were also visible on the TLC plate. The ELISAgram immunoblot procedure revealed that one of these spots, at R_f 0.2, cross-reacted with the anti-paxilline antibody. Consideration of the known cross-reactivity of this antibody[1] suggested that this spot was likely to be associated with a compound containing the intact rings A–E of paxilline.





The compound, which we name paxinorol (**2**), was isolated by flash chromatography as a colorless solid (1 mg). Because of the small quantity of **2** available, mp data were not obtained. Mass spectrometry revealed a prominent ion at *m/z* 182, consistent with the presence of an intact ring A–C system of paxilline.[4] The mass of the molecular ion was consistent with a molecular formula of $C_{24}H_{27}NO_3$. Acetylation gave a more mobile compound, the mass spectrum of which also showed a prominent ion at *m/z* 182 and a molecular ion consistent with a monoacetate with the molecular formula $C_{26}H_{29}NO_4$. Thus, **2** contained one primary or secondary hydroxyl group somewhere other than in rings A–C and a more-or-less intact ring A–E system of the type found in paxilline (**1**).

Because of the limited solubility of paxinorol in $CDCl_3$ or $(CD_3)_2CO$, the ^1H and ^{13}C NMR spectra of paxilline (**1**) and paxinorol (**2**) were obtained with C_5D_5N as the solvent. A complete assignment of the ^1H and ^{13}C NMR resonances of paxilline (**1**) was obtained (Table 1) by standard methodology.[3]

The ^{13}C NMR spectrum of paxinorol comprised nine quaternary, eight methine, five methylene, and two methyl carbons. Although the positions of the resonances

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Table 1. ^1H and ^{13}C NMR Assignments (δ) and $^1J(^{13}C-^1H)$ Coupling Constants (±1 Hz) for Paxilline (1) and Paxinorol (2) in C_5D_5N

	paxilline (1)				paxinorol (2)			
	^{13}C	$^1H\alpha$	$^1H\beta$	^1J-($^{13}C-^1H$)	^{13}C	$^1H\alpha$	$^1H\beta$	^1J-($^{13}C-^1H$)
2	153.6				154.0			
3	51.4				51.5			
4	43.4				43.5			
5	27.3	3.00	1.97		27.8	3.03	1.94	
6	29.0	2.25	1.96		28.7	2.36	1.74	
7	73.6	5.16		144.5	46.6	3.73		135.1
9	84.2	3.81		139.1	80.9		4.30	138.4
10	198.9				208.9			
11	119.6	5.14		163.9	121.7	5.99		169.1
12	169.9				183.4			
13	76.7				74.8			
14	33.9	1.95 −1.99[a]			33.7	1.97 −2.00[a]		
15	21.5	2.28	1.73		21.9	2.34	1.74	
16	50.2		3.01		50.4		3.03	
17	27.6	2.62	2.85		27.8	2.62	2.85	
18	116.6				116.5			
19	126.0				126.0			
20	118.7	7.29		156.9	118.7	7.24		156.4
21	119.4	7.24		156.9	119.4	7.21		156.9
22	120.2	7.74		156.9	120.2	7.71		157.2
23	112.4	7.53		156.9	112.4	7.56		156.7
24	141.3				141.3			
25	16.9	1.69		127.2	17.0	1.73		127.5
26	19.4	1.18		125.5	19.3	1.12		128.6
27	72.0							
28	25.6	1.52						
29	26.7	1.54						
13-OH		6.59				6.64		
9-OH		5.23				nd[b]		
NH		11.59				11.56		

[a] Broad multiplet [b] nd = not detected.

for C-2 to C-6 and C-14 to C-26 were virtually identical for **1** and **2**, those for C-7 to C-13 differed substantially. Furthermore, resonances attributable to the hydroxyisopropyl side chain present at C-9 of **1** (C-27 to C-29) were absent in **2**. Thus, with exception only of the ring F resonances, the majority of the ^{13}C NMR resonances of paxinorol were very similar to those for paxilline (**1**).

Differences between the ^1H NMR spectra of paxilline and paxinorol were also consistent with paxinorol being a tris-nor paxilline-like indole–diterpenoid. For example, four methyl signals appeared in the ^1H NMR spectrum of paxilline, but only two methyl resonances were present in the ^1H NMR spectrum of paxinorol. The absence of the methyl group signals attributable to the hydroxyisopropyl group of **1**, together with the 58 mass unit difference between paxilline and paxinorol, suggested that paxinorol might have been derived from paxilline by loss of a molecule of acetone, possibly via the retro-aldol reaction that is probably responsible for the formation of the ion at *m/z* 377 in the mass spectrum of paxilline. However, a more detailed analysis of ^1H and ^{13}C NMR data discounted the possibility that paxinorol was produced by a retro-aldol reaction of paxilline.

Notably, the two-dimensional $^{13}C-^1H$ correlated NMR spectrum of paxinorol revealed that the two ring F methine carbons (46.6 and 80.9 ppm) correlated with proton signals at 3.73 (H-7) and 4.30 (H-9) ppm, respectively. In the COSY spectrum, the proton signal at 3.73 ppm (H-7) correlated strongly with resonances at 2.36 (H-6α), 1.74 (H-6β), and 5.99 (H-11) ppm, respectively, and weakly to a signal at 4.30 ppm (H-9).

The foregoing ^1H and ^{13}C NMR data indicate that C-9 of paxinorol is oxygenated, but that C-7 is not. In particular, the C-7 and H-7 resonances of paxilline occur

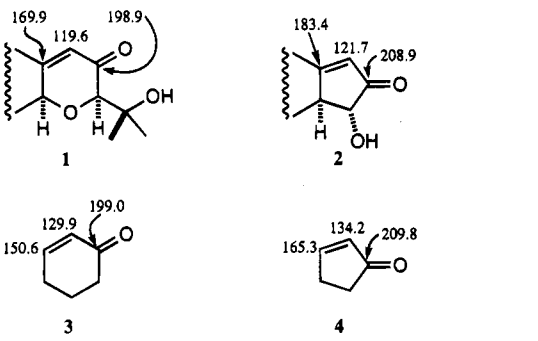

Figure 1. ^{13}C NMR chemical shifts for C-10 to C-12 of **1** and **2** and for the equivalent resonances of 2-cyclohexen-1-one (**3**) and 2-cyclopenten-1-one (**4**) (data for **3** and **4** from ref 5).

at 73.6 and 5.16 ppm, respectively, whereas the equivalent resonances of paxinorol (**2**) occur at 46.6 and 3.73 ppm. On the other hand, the C-9 (80.9 ppm) and H-9 (4.30 ppm) resonances of **2** are similar to those of **1** (84.2 and 3.81 ppm, respectively), consistent with oxygenation at C-9. Paxinorol (**2**) therefore lacks the ether bridge present at O-8 of **1**. These observations suggested the presence in paxinorol of a direct C-7–C-9 bond and a hydroxyl group at C-9, with a five-membered ring F. The coupling constant exhibited by H-9 (J = 3.2 Hz), which the COSY spectrum indicated to arise from coupling with H-7 (see above), was also consistent with a ^3J coupling (rather than a ^4J coupling) between H-7 and H-9.

Comparison (Figure 1) of the C-10 and C-12 resonances established for paxinorol (**2**) (208.9 and 183.4 ppm, respectively) and for paxilline (**1**) (198.9 and 169.9 ppm, respectively) with those reported[5] for the C-1 and C-3 resonances of 2-cyclopenten-1-one (**4**) (209.8 and 165.3 ppm, respectively) and 2-cyclohexen-1-one (**3**) (199.0 and 150.6 ppm, respectively) supports this proposal, because the downfield shifts experienced by C-1 (+10.8 ppm) and C-3 (+14.7 ppm) of **4**, relative to **3**, are analogous to those exhibited by C-10 (+10.0 ppm) and C-12 (+13.5 ppm) of **2**, relative to **1**.

Additionally, the $^1J(^{13}C-^1H)$ coupling constants determined for C-7 (135.1 Hz) and C-9 (138.4 Hz) in paxinorol are consistent with the proposed structure **2**, the equivalent $^1J(^{13}C-^1H)$ coupling constants for paxilline being 144.5 Hz and 139.1 Hz, respectively. Comparison of the C-11 ^1J coupling constants of paxinorol (169.1 Hz) and paxilline (163.9 Hz) also suggests a five-membered cyclopentenone-type ring system in paxinorol (**2**), rather than a six-membered F-ring such as is present in paxilline (**1**), because it is well established that in small ring systems a decrease in ring size is accompanied by an increase in coupling constant—an effect ascribed to the increased s-character of the hybrid carbon orbitals in the ring.[5]

The stereochemistry at C-9 of paxinorol was established in NOE difference experiments. Irradiation of H-9 (4.30 ppm) caused NOE enhancement of H-6β (3.9%) and H-7α (2.1%), whereas irradiation of H-6β (1.74 ppm) enhanced H-6α (8.1%), H-26 (i.e. the 4β-Me group) (1.0%), and H-9 (2.0%). Irradiation of H-7α (3.73 ppm) enhanced the H-9 (2.4%), H-5α (1.0%), H-6α (3.9%), and 13-OH (2.6%) resonances, whereas irradiation of H-6α (2.36 ppm) enhanced H-6β (18.5%) and H-7α (4.4%), but not

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H-9. It follows from those observations that H-9 is β-oriented and that the 9-hydroxyl group is therefore α-oriented. The observation of an NOE between H-9 and H-6β is of paramount importance in defining the C-9 stereochemistry of paxinorol, because molecular models clearly demonstrated that had H-9 been α-oriented it would have been too distant from H-6β for an NOE to have been observed.

Because the resonances of H-6α and H-6β were coincident with those of H-15α and H-15β, respectively, the preceding NOE experiments also resulted in enhancements of suitably oriented ring C/D protons. Fortunately, none of the NOEs originating from H-15α, or from H-15β, interfered with the detection of the NOEs originating from H-6α, H-6β, H-7α, and H-9, because all of these protons are too distant from H-15α and H-15β for mutual NOEs to be observed.

The structure of paxinorol is therefore established as **2**, which is formally derived from **1** by loss of acetone. It is not possible to be certain that **2** is a natural product because, due to the prolonged storage prior to its isolation, it may have been produced abiotically from the much more abundant **1**. Nevertheless, the identification of **2** by TLC–ELISAgram, and its subsequent isolation, illustrates the considerable potential that exists for use of this and other immunochemical techniques in natural products chemistry.

Experimental Section

General Methods. TLC was performed on plates coated with silica gel 60 F_{254} (0.2 mm) with CH_3OH–$CHCl_3$ (1:19) as the eluent, and compounds were detected by their fluorescence-quenching upon irradiation at 254 nm. Flash column chromatography[6] was performed on silica gel with CH_3OH–CH_2Cl_2 (1:24 for paxinorol; 1:49 for paxinorol acetate) as the eluent. Paxinorol was acetylated with acetic anhydride–pyridine (1:1, 1 mL) overnight before removal of the solvent in vacuo (toluene azeotrope) and purification by column chromatography. *P. paxilli* (FRR1900) was grown in culture and crude paxilline extracted from the mycelium as previously described.[7] This extract was defatted by partitioning between petroleum spirit and ethanol–water (1:4), and the ethanolic extract was evaporated to dryness in vacuo. The extract was stored in the dark at 4 °C for 1 year prior to isolation of **2**. Authentic paxilline was available from earlier work in our laboratory.[3] The TLC–ELISAgram was obtained by the method of Pestka,[2] with a monoclonal anti-paxilline antibody generated by immunization of mice with conjugates of paxilline *O*-(carboxymethyl)oxime.[1] Briefly, a nitrocellulose sheet was coated with antibody by incubating it with the anti-paxilline antibody overnight, and additional protein-binding sites were blocked by incubation (1 h) with 1% bovine serum albumin. The developed TLC plate was air-dried and contact-blotted against the antibody-coated nitrocellulose sheet. After capture of cross-reacting material by the antibody, the nitrocellulose sheet was developed by incubation with a paxilline–enzyme conjugate. Incubation with the substrate then revealed areas of the sheet containing cross-reacting compounds as colorless regions against a uniformly colored background.

Nuclear Magnetic Resonance Spectroscopy. ^1H and ^{13}C NMR spectra were obtained at 300 K from C_5D_5N solutions unless otherwise stated, at 300 and 75 MHz, respectively. Carbon signal multiplicities (s, d, t, or q) were determined using the DEPT135 sequence. NOE difference experiments were performed with an irradiation power level of 40 L. NOE difference spectra were obtained by subtraction of an off-resonance control FID from an irradiated FID and Fourier transformation of the resulting difference-FID. Two-dimensional COSY and double quantum-filtered COSY spectra were determined in absolute value mode, and the ^{13}C–^1H-correlated spectrum was determined in phase-sensitive mode.

Paxinorol (2). Paxinorol was isolated as a colorless solid: ^1H NMR δ 1.12 (H-26), 1.73 (H-25), 1.64–1.79 (m, H-6β and H-15β), 1.88–2.05 (m, H-5β, H-14α, and H-14β), 2.29–2.43 (m, H-6α and H-15α), 2.62 (dd, J = 13.0, 11.0 Hz, H-17α), 2.85 (dd, J = 13.0, 6.1 Hz, H-17β), 2.93–3.10 (m, H-5α and H-16β), 3.73 (br t, J = 10.6 Hz, H-7), 4.30 (d, J = 3.2 Hz, H-9), 5.99 (br d, J = 1.8 Hz, H-11), 6.64 (s, 13-OH), 7.21–7.24 (m, H-20 and H-21), 7.56 (m, H-23), 7.71 (m, H-22), 11.56 (br s, NH); ^{13}C NMR and ^{13}C–^1H two-dimensional correlations, see Table 1; EI-MS *m/z* (rel intensity) 377.1966 (M^+, calcd *m/z* 377.1992 for $C_{24}H_{27}NO_3$, 6), 362 (100), 344 (59), 182 (97). ^1H and ^{13}C NMR assignments for **2** are reported in Table 1.

Paxinorol 9-acetate, a colorless oil, was prepared by acetylation of **2**: ^1H NMR ($CDCl_3$) δ 1.02 (H-26), 1.37 (H-25), 2.16 (CH_3COO), 4.91 (d, J = 3.2 Hz, H-9), 5.89 (br d, J = 1.7 Hz, H-11), 7.08 (m, H-21 and H-22), 7.30 (m, H-23), 7.44 (m, H-20), 7.72 (br s, NH); EI-MS *m/z* (rel intensity) 419.2128 (M^+, calcd *m/z* 419.2098 for $C_{26}H_{29}NO_4$, 6), 404 (78), 386 (53), 182 (100).

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Supporting Information Available: ^{13}C NMR spectra of **1** and **2** and cross-reactivity data for the anti-paxilline antibody (4 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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