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ISOLATION OF PASPALINE B, AN INDOLE-DITERPENOID FROM *PENICILLIUM PAXILLI*

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Key Word Index—*Penicillium paxilli*; biosynthesis; indole–diterpene; tremorgen; paxilline; 13-desoxypaxilline; paspaline; paspaline B.

Abstract—The known indole–diterpenoids paspaline and 13-desoxypaxilline were isolated from *Penicillium paxilli* Bainier for the first time. In addition, paspaline B, a new indole–diterpenoid, was isolated and identified as paspalin-30-al by a combination of mass spectral and 1D and 2D NMR techniques. Full assignments for the ^{13}C NMR chemical shifts of paspaline and paspaline B were obtained, resulting in a major revision of the published ^{13}C NMR chemical shifts for paspaline. The structure of paspaline B suggests that it may closely follow paspaline in the biosynthesis of the tremorgenic indole–diterpenoid paxilline.

INTRODUCTION

The lolitrems are potent tremorgenic mycotoxins produced by *Acremonium lolii* and are thought to be the causative agents of the neurotoxic disease of livestock known as perennial ryegrass staggers [1–4]. Paxilline (**1**) and α- and β-paxitriols are thought to be crucial intermediates in the biosynthesis of the lolitrems [2,4–8], as well as of a number of other tremorgenic mycotoxins [7,9]. During a large-scale reduction of **1**, isolated from extracts of *Penicillium paxilli* Bainier, we observed the presence of a number of congeners (to be reported elsewhere). This finding prompted a detailed examination of the paxilline for contaminants, resulting in the identification of the known indole–diterpenoids 13-desoxypaxilline (**2**) and paspaline (**3**) and of a novel indole–diterpene which we name paspaline B (**4**).

RESULTS AND DISCUSSION

When apparently pure [2] paxilline, isolated from *P. paxilli* extracts, was recrystallized the resulting mother liquor was found to be enriched in minor components which had been present as trace contaminants of the original paxilline. In addition to paxilline, HPLC revealed the presence in the mother liquor of three compounds, each of which possessed a UV absorbance spectrum similar (Fig. 1) to that of paxilline [10]. These compounds were isolated by semi-preparative HPLC. Comparison of their NMR and mass spectral properties with those in the literature revealed two of these compounds to be the known indole–diterpenoids 13-desoxypaxilline (**2**) [11–13] and paspaline (**3**) [11,13–17].

The HR mass spectrum of the third compound (**4**) indicated it to have the formula $C_{28}H_{37}NO_3$, consistent with an oxidized form of paspaline. However, the ^1H and ^{13}C NMR spectra of **4** contained only four resonances attributable to methyl groups, suggesting that **4** was an analogue of **3** in which one of the five methyl groups had been oxidized to an aldehyde group. The mass spectrum of **4** included a strong ion at *m/z* 182, consistent with the presence in **4** of the intact rings A–C of **1** [7]. Comparison of the 1D and 2D NMR spectral data determined for **3** and **4** (Tables 1 and 2) revealed that it was the methyl group on C-12 (i.e. C-30) which was oxidized in paspaline B; paspaline B was thus identified as paspalin-30-al (**4**). The long-range ^2J and ^3J ^{13}C–^1H-correlated NMR data presented in Table 2 demonstrate that methyl groups on C-3 and C-4 (H-25 and H-26) are present in both **3** and **4** because, for each of these compounds, their H-25 and H-26 (methyl) resonances exhibited correlations with the C-3 and C-4 resonances, respectively. Consistent with the introduction of the aldehyde functionality at C-30, C-12 of **4** resonates at 50.7 ppm, whereas the equivalent resonance of **3** occurs at 36.6 ppm (Table 1). Moreover, irradiation of the aldehydic proton resonance (H-30) of **4** caused enhancement of the methyl resonance at 0.94 ppm (H-26). The reciprocal NOE was also observed, thereby establishing a *cis*-relationship between C-30 and C-26 in **4**.

Cross-peaks observed in the HMBC spectrum of **4** (Table 2) identified the C-27 (71.9 ppm) and C-9 (85.0

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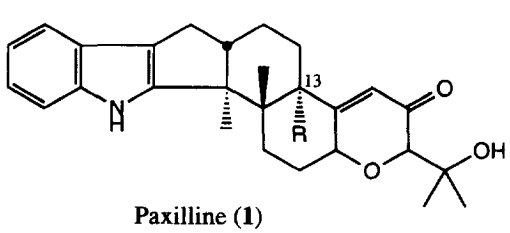
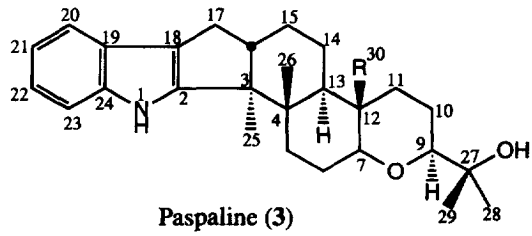


Paxilline (**1**)
13-Desoxypaxilline (**2**)

R	
1	OH
2	H



Paspaline (**3**)
Paspaline B (**4**)

R	
3	Me
4	CHO

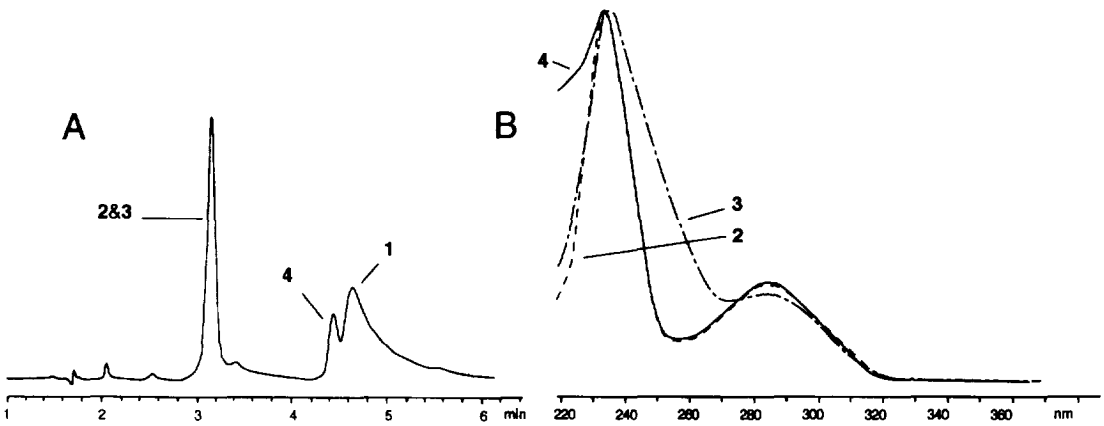


Fig. 1. (A) HPLC chromatogram of the mother liquor generated by recrystallization of apparently pure paxilline. (B) UV absorbance spectra obtained from chromatograms of purified specimens of 13-desoxypaxilline (**2**), paspaline (**3**) and paspaline B (**4**) by means of a diode-array detector.

ppm) resonances from their ^2J and ^3J couplings with the C-27 methyl protons (H-28 and H-29), and the C-7 (83.6 ppm)–H-7 (3.32 ppm) and C-9 (85.0 ppm)–H-9 (3.28 ppm) connectivities were revealed in the HMQC spectrum. Cross-peaks observed in the COSY spectrum of **4** then identified the H-9–H-10α/H-10β–H-11α/H-11β, and H-7–H-6α/H-6β–H-5α/H-5β spin systems. This enabled the C-10 (23.4 ppm), C-11 (33.9 ppm), C-6 (24.9 ppm) and C-5 (33.2 ppm) methylene carbon resonances to be assigned from cross-peaks appearing in the HMQC spectrum. It was also apparent from the HMQC spectrum that H-17α (2.32 ppm) and H-17β (2.68 ppm) correlated with the methylene carbon resonance at 27.4 ppm. These observations, along with the complete assignment of the ^{1}H and ^{13}C NMR resonances of **3** (see below), enabled us to derive the ^{1}H and ^{13}C NMR assignments for **4** given in Table 1.

In their pioneering investigation of the biosynthesis of paspaline, Acklin *et al.* [18] employed ^{13}C NMR spectroscopy to determine the sites of incorporation of ^{13}C label in paspaline produced by *Claviceps paspali* when fed a variety of ^{13}C-labelled precursors. However, no ^{13}C NMR assignments were reported [18], and interchangeable assignments have subsequently been advanced for many of the carbon resonances, especially the methylene resonances, of paspaline. A common feature of many of the published assignments [11, 13, 15] is the proposition that the methylene carbon resonance at 34 ppm in **3** arises from C-17. In the course of our structure elucidation of **4** (see above), it became apparent this resonance arose from C-5, and that significant revisions of the published assignments of **3** were therefore required.

Careful analysis of the COSY spectrum of **3** identified three independent spin correlation pathways (cross-peak connectivities) incorporating all of the methylene protons, namely: H-7–H-6α/H-6β–H-5α/H-5β; H-9–H-10α/H-10β–H-11α/H-11β; and H-17α/H-17β–H-16–H-15α/H-15β–H-14α/H-14β–H-13. The orientation of individual methylene protons (α or β) was established from a combination of NOE-difference results and (where resolved) coupling constant data. Cross-peaks observed in the phase-sensitive ^{13}C–^{1}H correlated spectrum of **3** readily identified the corresponding methylene carbon resonances; furthermore, the resolution in the ^{1}H axis of the spectrum was such that large ^2J and axial–axial ^3J couplings (*ca* 10–14 Hz) were resolved, whereas smaller ^3J axial–equatorial or equatorial–equatorial proton couplings were not [2]. For example, H-5β and H-11β

Table 1. ^{13}C and ^{1}H NMR chemical shifts (δ, $CDCl_3$) determined for paspaline (3) and paspaline B (4)

	3		4	
Atom	^{13}C	^{1}H*	^{13}C	^{1}H*
2	150.9		150.0	
3	53.0		52.0	
4	40.1		39.9	
5	34.0	1.96, 1.63	33.2	2.07, 1.72
6	24.7	1.72, 1.82	24.9	2.04, 2.24
7	85.8	3.04	83.6	3.32
9	84.8	3.23	85.0	3.28
10	22.0	1.45, 1.66	23.4	1.29, 1.55
11	37.7	1.14, 1.85	33.9	1.22, 2.49
12	36.6		50.7	
13	46.5	1.50	47.6	1.69
14	22.0	1.46, 1.69	22.4	1.55†, 1.90
15	25.3	1.61, 1.79	24.9	1.55†, 1.80
16	48.8	2.76	48.7	2.70
17	27.6	2.34, 2.68	27.4	2.32, 2.68
18	118.3		118.0‡	
19	125.2		125.7‡	
20	118.4	7.43	118.6	7.41
21	119.6	7.08	119.7	7.07
22	120.5	7.08	120.7	7.07
23	111.5	7.30	111.5	7.29
24	140.0		140.0	
25	14.6	1.03	14.8	1.01
26	20.0	1.14	19.7	0.94
27	72.0		71.9	
28	23.8	1.19	23.8	1.12
29	26.1	1.21	25.8	1.17
30	12.7	0.88	207.3	10.13
NH		7.81		7.69

*Methylene proton resonances in the format [Hα, Hβ].

†Overlapping multiplets (1.5–1.6 ppm); individual chemical shifts not assigned.

‡Tentative assignments due to limited signal:noise.

afforded doublet-like cross-peaks, and H-5α and H-11α afforded triplet-like cross peaks. The assignments given in Table 1 were further corroborated by cross-peaks observed in the long-range HMBC spectrum (Table 2).

These results reveal that nine of the 30 ^{13}C resonances in **3**, including four resonances given 'interchangeable assignments', have been incorrectly assigned in the literature [11, 13, 15]. In particular, our NMR spectral analysis revealed that the C-17 resonance of **3** occurs at 27.6 ppm, rather than at 34.0 ppm. That the C-14 resonances of **3** and **4** occur at 22–23 ppm, compared to 29–35 ppm in the penitrems [19–21], the janthitrems [22–24], paxilline and lolitrem derivatives [2–4], can be attributed to the absence in **3** and **4** of a hydroxyl group at C-13 and the consequent absence of a β-substituent group effect of *ca* +3.7 to +7.4 ppm [25].

Paspaline [7, 11, 18, 26–28] and 13-desoxypaxilline [7, 11] have been proposed as biosynthetic intermediates of the indole–diterpenoid tremorgens. Paspaline and 13-desoxypaxilline have not previously been reported in the genus *Penicillium*, and their identification as metabolites of *P. paxilli* is fully consistent with a recently proposed metabolic grid [7].

Paspaline has been shown to be a precursor of more complex indole–diterpenoids [26], presumably by loss of the methyl at C-12 to give an 11,12-unsaturated intermediate (such as the hypothetical alkene **5** in Fig. 2). This intermediate could then be oxidized to known compounds such as PC-M6 [21], and on to compound **2**. Compound **4** is the first oxidized analogue of paspaline to be isolated. The structure of **4** suggests that it may represent one of the early steps in the demethylation of paspaline to form the C_{27} unit (i.e. **5**) on which all the complex indole–diterpenoid tremorgens (e.g. paxilline, the penitrems, janthitrems and lolitrems) are based. Formal loss of formaldehyde from **4** would give the 11,12-unsaturated C_{27} unit (**5**); alternatively, **5** could be generated by further oxidation of **4** at C-30 to give paspalin-30-oic acid, followed by formal loss of formic acid.

The recent isolation from *Emericella nivea* of emeniveol [29], a potential biosynthetic precursor of emindole SB, appears to clarify further the probable early stages in the biosynthesis of the indole–diterpenoids.

We therefore propose (Fig. 2) modifications to the early stages of the metabolic grid for the indole–diterpenoids, as originally proposed by Mantle and Weedon [7]. The validity of their scheme continues to gather support from the finding of key intermediates, such as paspaline and 13-desoxypaxilline, across several indole–diterpenoid-producing genera (e.g. paspaline in *Albophoma* [30], *Claviceps* [14], *Emericella* [11, 13] and *Penicillium*; 13-desoxypaxilline in *Emericella* [11, 13] and *Penicillium*; penitrems in *Aspergillus* [31] and *Penicillium* [19, 20]; and paxilline in *Acremonium* [5], *Emericella* [12] and *Penicillium* [32]).

The application of modern analytical tools, such as HPLC coupled with diode-array UV detection [3, 4, 10], and the advent of antibodies with wide cross-reactivity against groups of indole–diterpenoids [33], will greatly facilitate the isolation of new indole–diterpenoids. Indeed, a new analogue of paxilline has recently been isolated from *P. paxilli* [34] with the aid of TLC-ELISAgram methodology [35]. Furthermore, the compounds identified in the present study may well have been the source of some of the unidentified peaks in a chromatogram of a *P. paxilli* extract that was published in a recent paper illustrating the utility of diode-array detection in indole–diterpenoid analysis [10].

The only toxicosis of major economic importance that is believed to be caused by tremorgenic indole–diterpenoids is perennial ryegrass staggers. This disease is attributed to the production by the endophytic fungus *A. lolii*, which infects much of the world's perennial ryegrass (*Lolium perenne*), of tremorgenic neurotoxins (paxilline and the lolitrems) [1, 2, 4, 5, 8]. The rapid improvement in our understanding of indole–diterpenoid biosynthesis may eventually allow rational screening of *Acremonium* endophytes for strains incapable of tremorgen production. Such strains might then be inoculated into ryegrasses to generate cultivars possessing the beneficial

Table 2. Long range ^2J and ^3J ^{13}C–^1H correlations observed in the 2D HMBC NMR spectra of paspaline (3) and paspaline B (4)

^1H signal (δ ppm)	Correlated ^{13}C signals (δ ppm)
Paspaline (3)	
0.88 (H-30)	85.8 (C-7), 46.5 (C-13), 37.2 (C-11 and C-12)*
1.03 (H-25)	150.9 (C-2), 53.0 (C-3), 48.8 (C-16), 40.1 (C-4)
1.14 (H-26)	53.0 (C-3), 46.5 (C-13), 40.1 (C-4), 34.0 (C-5)
1.19 (H-28)	84.8 (C-9), 72.0 (C-27), 23.8 (C-28)
1.21 (H-29)	84.8 (C-9), 72.0 (C-27), 26.1 (C-29)
2.34 (H-17α)	150.9 (C-2), 118.3 (C-18), 48.8 (C-16), 25.3 (C-15)
2.68 (H-17β)	150.9 (C-2), 118.3 (C-18), 53.0 (C-3), 48.8 (C-16)
3.04 (H-7)	84.8 (C-9), 37.7 (C-11), 24.7 (C-6), 12.7 (C-30)
3.23 (H-9)	85.8 (C-7), 72.0 (C-27), 26.1 (C-29), 23.8 (C-28)
7.81 (NH)	150.9 (C-2), 140.0 (C-24), 125.2 (C-19), 118.3 (C-18)
Paspaline B (4)	
0.94 (H-26)	52.0 (C-3), 47.6 (C-13), 39.9 (C-4), 33.2 (C-5)
1.01 (H-25)	150.0 (C-2), 52.0 (C-3), 48.7 (C-16), 39.9 (C-4)
1.12 (H-28)	85.0 (C-9), 71.9 (C-27), 25.8 (C-29)
1.17 (H-29)	85.0 (C-9), 71.9 (C-27), 23.8 (C-28)

*Broad signal considered to arise from overlap of the C-11 (37.7 ppm) and C-12 (36.6 ppm) resonances.

effects of endophyte infection, but incapable of causing the neurotoxic syndrome ryegrass staggers.

EXPERIMENTAL

General. MS: direct insertion probe on a Kratos MS-80 RFA instrument; flash CC: silica gel (Merck, Art. 9385). The paxilline content of frs obtained during purification was assessed by HPLC on a 4.6 mm × 25 cm, 5 μm Zorbax silica gel column with MeCN–CH_2Cl_2 (1:4) as eluent (1.8 ml min^{-1}), and eluting compounds were detected with a Hewlett-Packard 1040M diode-array UV detector. Semiprep. HPLC purification was performed on an RCM-100 Radial Compression Separation System (Waters) fitted with a silica gel Radial-PAK cartridge (8 mm × 10 cm, 10 μm) (Waters), with MeCN–CH_2Cl_2 (3:17) as eluent (3.0 ml min^{-1}). Eluting compounds were detected with an LC-85B spectrophotometric detector (Perkin-Elmer). Authentic paxilline [2] and paspaline [36] were available from earlier investigations.

NMR. 1D and 2D ^1H (300.13 MHz) and ^{13}C (75.47 MHz) NMR spectra were determined at 300 K on a Bruker AC-300 instrument fitted with a standard 5 mm probe head. Resolution enhancement (LB = −1.5, GB = 0.33) was applied to some of the ^1H NMR spectra to assist in determination of coupling constants. ^{13}C NMR signal multiplicities (*s*, *d*, *t* or *q*) were determined with the DEPT135 sequence. NOE-difference experiments were performed with an irradiation power of 45 L (methyl resonances) or 40 L (low-field proton resonances). 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. 2D COSY and inverse-mode HMBC spectra were determined in absolute value mode, whereas inverse-mode HMQC spectra, optimized for detection of one-bond ^{13}C–^1H couplings, were determined in phase-sensitive mode.

Production and purification of paxilline. Paxilline was produced by inoculating Roux flasks, each containing Weetbix (25 g), Difco mycological broth (50 ml), yeast extract (1 g), sucrose (10 g) and H_2O (200 ml), with *P. paxilli* (ATCC 26601). Fungal cultures were incubated for 3 weeks at 25°. Once the incubation period was complete, the fungal cultures were added to $CHCl_3$ (300 ml) and homogenized in a Sorvall Omnimixer. The mixt. was then centrifuged, and the $CHCl_3$ layer recovered. The $CHCl_3$ soln was dried ($MgSO_4$) and the solvent removed *in vacuo*. The resulting solid was dissolved in petrol (40–60°) (1 l) and partitioned against EtOH–H_2O (4:1, 1 l). The alcoholic extract was diluted by addition of H_2O (1 l), extracted with CH_2Cl_2 (2 × 1 l), and the solvent removed *in vacuo* from the dried ($MgSO_4$) extracts. The resulting brown solid was purified by flash CC, with elution by a step-wise gradient of MeCN–CH_2Cl_2 (1:19, 1 l; 1:9, 1 l; 3:17, 1 l; 1:4 1 l 3:7 1 l) and 0.5 l frs were collected. Frs containing paxilline were identified by HPLC and concd *in vacuo*. Crystallization from MeCN–CH_2Cl_2 gave paxilline as a solid, identical by ^1H and ^{13}C NMR, EI-MS, TLC and HPLC to authentic paxilline [2].

Isolation of paspaline, 13-desoxypaxilline and paspaline B. Recrystallization of the 'pure' paxilline (3 g), prepd as described above, from MeCN–CH_2Cl_2, gave pure paxilline. The mother liquor from this crystallization was examined by HPLC and found to contain, in addition to paxilline, a number of other compounds with UV spectra similar to that of paxilline (λ_{max} at 230 and 280 nm).

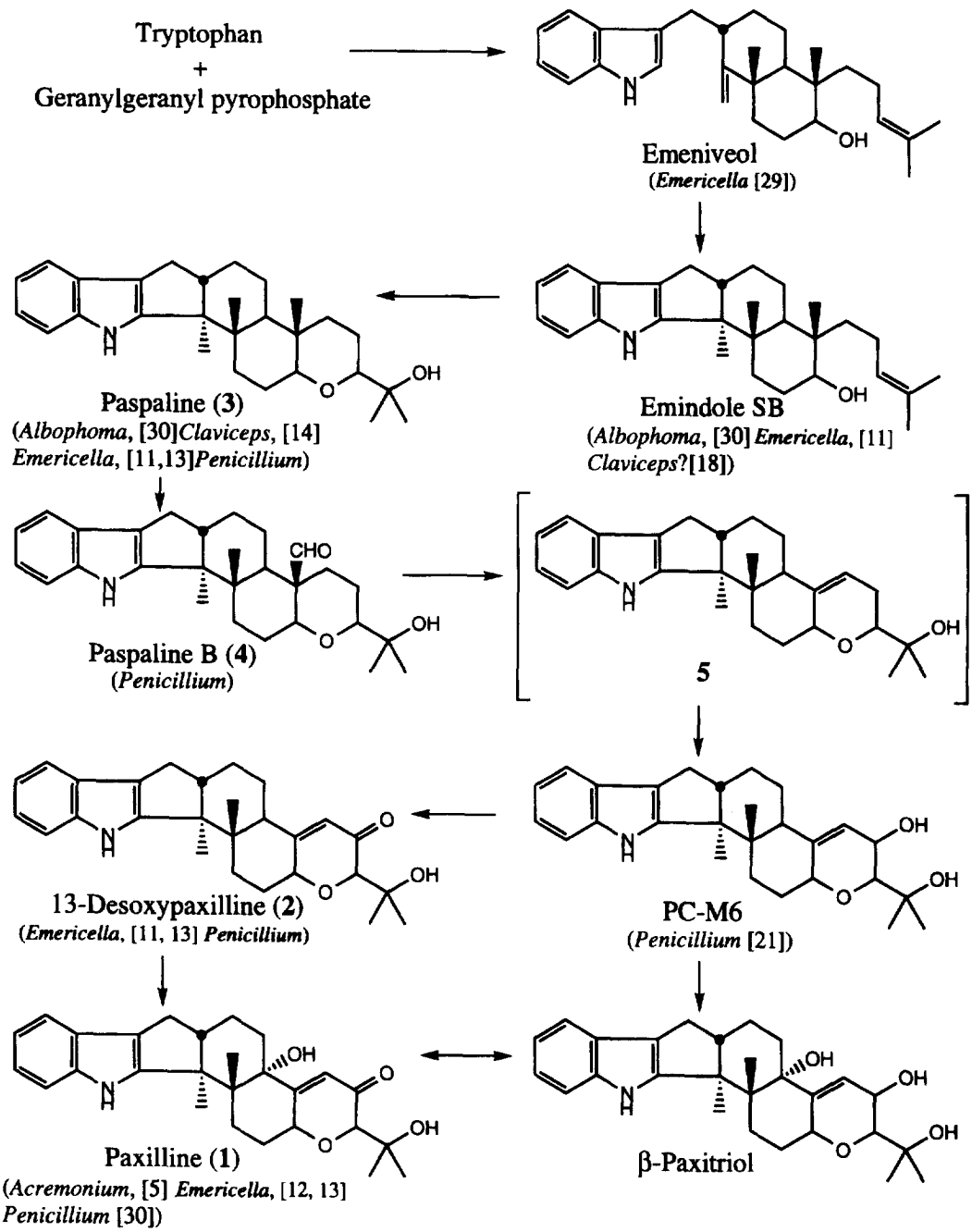


Fig. 2. Proposed metabolic grid, adapted from that of Mantle and Weedon [7], for the early stages of indole–diterpenoid biosynthesis, showing possible roles for the compounds **1–4** isolated in this study. Genera from which each compound has been isolated are given in parentheses below the compound's name.

Semiprep. HPLC of the mother liquor gave 2 pure compounds and a mixed fr. The pure compounds were identified from their published [11–17] NMR and mass spectra as 13-desoxypaxilline (**2**). ^{1}H NMR (δ $CDCl_3$): 1.00 (3H, *s*, H-25), 1.08 (3H, *s*, H-26), 1.28 (3H, *s*, H-28), 1.29 (3H, *s*, H-29),2.42 (1H, *dd*, J = 13.0, 10.2 Hz, H-17α), 2.74 (1H, *dd*, J = 13.0, 6.3 Hz, H-17β), 2.81 (1H, *m*, H-16), 3.73 (1H, *d*, J = 2.1 Hz, H-9), 4.21 (1H, *br s*, 27-OH), 4.27 (1H, *br t*, J = 8.8 Hz, H-7α), 5.88 (1H, *br s*, H-11), 7.09 (2H, *m*, H-21 and H-22), 7.31 (1H, *m*, H-23), 7.4 (1H, *m*, H-24), 7.76 (1H, *br s*, NH); EIMS *m/z* (rel. int.): 419.2460 $[M]^+$ (419.2462 for $C_{27}H_{33}NO_3$, 7%), 404 (9), 386 (10), 362 (16), 361 (59), 347 (25), 346 (100), 256 (9), 182 (44), 168 (21), 129 (22), and paspaline (**3**) ^{1}H NMR (δ $CDCl_3$): 0.88 (3H, *s*, H-30), 1.03 (3 H, *s*, H-25), 1.14 (3H, *s*, H-26), 1.19 (3H, *s*, H-28), 1.21 (3H, *s*, H-29), 2.34 (1H, *dd*, J = 13.0, 10.4 Hz, H-17α), 2.68 (1H, *dd*, J = 13.0, 6.4 Hz, H-17β), 2.76 (1H, *m*, H-16), 3.04 (1H, *dd*, J = 11.4, 4.0 Hz, H-7α), 3.23 (1H,

dd, J = 11.9, 3.1 Hz, H-9), 7.08 (2H, *m*, H-21 and H-22), 7.30 (1H, *m*, H-23), 7.43 (1H, *m*, H-20), 7.81 (1H, *br s*, NH); ^{13}C NMR: Table 1; EIMS *m/z* (rel. int.): 421.2981 $[M]^+$ (421.2983 for $C_{28}H_{39}NO_2$, 41), 403 (76), 389 (30), 388 (96), 182 (100), 168 (20), 144 (12), 130 (30).

Semiprep. HPLC of the mixed fr. yielded a 3rd compound, paspaline B (**4**), as a solid. ^{1}H NMR (δ $CDCl_3$): 0.94 (3H, *s*, H-26), 1.01 (3H, *s*, H-25), 1.12 (3H, *s*, H-28), 1.17 (3H, *s*, H-29), 2.64–2.72 (2H, *m*, H-16, H-17β), 3.32 (1H, *dd*, J = 11.7 , 2.5 Hz, H-7α), 3.28 (1H, *dd*, J = 12.4, 4.5 Hz, H-9), 7.07 (2H, *m*, H-21, H-22), 7.29 (1H, *m*, H-23), 7.41 (1H, *m*, H-20) 7.69 (1H , *br s*, NH), 10.13 (1H, *s*, H-30); ^{13}C NMR: Table 1; EIMS *m/z* (rel. int.): 435.2776 $[M]^+$ (435.2775 for $C_{28}H_{37}NO_3$, 88), 420 (73), 402 (27), 392 (20), 374 (40), 332 (17), 279 (24), 182 (75), 167 (28), 149 (58), 130 (100).

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