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Molecules of Interest

The role of tryptophan as a biosynthetic precursor of indole-diterpenoid fungal metabolites: Continuing a debate

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ABSTRACT

Studies in the 1980s and 1990s on the origin of the indole moiety in fungal indole-diterpenoids using ^{14}C-labelled tryptophan consistently showed autoradiographic evidence but gave low % incorporation of the probe. Recent studies on a member of the group (nodulisporic acid A), using more specific ^{13}C methodology, demonstrated a role of the tryptophan biosynthetic pathway but, in failing to show involvement of end-product, concluded that the indole was derived from indole-3-glycerol phosphate and suggested that the previous ^{14}C data arose *via* metabolic scrambling of label. In considering the protocol for the ^{13}C studies, there is concern that the fungal material was starved of an exogenous nitrogen source and thus could have degraded added labelled tryptophan. Consequently, synthesis of the serine necessary for anabolic formation of tryptophan may have been constrained. It is suggested that ^{13}C studies on appropriate fungi early in the idiophase of submerged or surface fermentation should be made before the biosynthesis of indole-diterpenoids can become clearer.

1. Landmarks in indole-diterpenoid discovery

Recognition of the structural class of indole-diterpenoid fungal metabolites has resulted from characterisation of several bioactive fungal metabolites since the 1970s. Interest from a mycotoxicological point of view had already been raised in the 1960s by the discovery (Wilson and Wilson, 1964; Wilson et al., 1968) of tremorgenic effects, subsequently shown to be caused by the *Aspergillus* product aflatrem (**1**) (Gallagher et al., 1980) and the *Penicillium* product penitrem A (**2**). Neurotoxicity in dogs, associated with autumn browsing on mouldy walnuts, was attributed to penitrem A (**2**) (Richard et al., 1981) and, together with a possible human case (Cole et al., 1983), ensured serious attention to these new compounds. Concurrently, two notable so-called 'staggers' syndromes had been economically troublesome in pastoral ruminants. Paspalum staggers was associated mainly with cattle in tropical latitudes where *Paspalum* grasses and their associated ergot fungus pathogen *Claviceps paspali* are endemic. Ryegrass staggers was notably associated with both sheep and cattle grazing ryegrass-dominant pastures during summer months in New Zealand (http://www.merinoinc.co.nz/Reports/Ryegrass%20Endophyte%20report.pdf). Paspalum staggers had long been thought to be due to the sclerotia of *C. paspali* located *in situ* in inflorescences grazed as rank native forage (*Paspalum dilatatum*), or as the more compact *P. distichum*, in their native South America or where introduced into the New World.

Use of the potently-tremorgenic compound penitrem A (**2**) in experimental modelling of ryegrass staggers symptomatology at the Ruakura Agricultural Research Centre in New Zealand in the mid 1970s demonstrated symptoms strikingly-similar to ryegrass staggers (Mantle et al., 1978). However, it was difficult to demonstrate sufficient natural intake *via* grazing (Mantle et al., 1978; Di Menna and Mantle, 1978). Perceptive interpretation of a spontaneous occurrence of ryegrass staggers in pasture productivity studies at Lincoln, New Zealand in 1980 sharpened focus on the cryptic ryegrass endophyte that had long been regarded as an insignificant fungal commensal (Fletcher and Harvey, 1981). Further direct experiments confirmed that the endophyte was the critical biological factor (Gallagher et al., 1981). There followed a cascade of discoveries, namely the seminal publication of penitrem structures (de Jesus et al., 1883) which set a model for the structural group, the structural characterisation of the ryegrass endophyte's indole-diterpenoid trace metabolite lolitrem B (**3**) as the disease determinant (Gallagher et al., 1984), description of the endophyte *Neotyphodium lolii* as closely related to ergot fungi, and the characterisation of paspaline (**4**) as a key tremorgenic agent in the ergot sclerotia causing Paspalum staggers (Cole et al., 1977). The ryegrass endophyte was also found to produce the ergot alkaloid ergovaline (**5**) which, like all other ergoline alkaloids, is an indole-isoprenoid derived from tryptophan (**6**). Until recently, there

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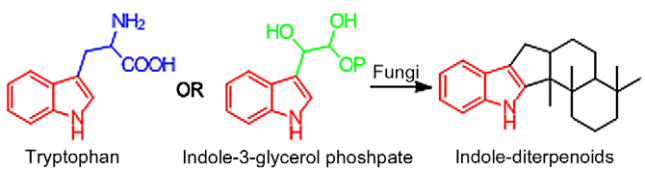
has been no suggestion that the indole moiety of indole-diterpenoids might be derived other than from tryptophan (**6**). However, a recent addition to the indole-diterpenoid group is nodulisporic acid (**7**) (Hensens et al., 1999), a metabolite of another fungal endophyte (*Nodulisporium* sp.); re-appraisal of evidence for biosynthesis of its indole moiety is the reason for the present communication (Scheme 1). Meanwhile topically, an indole-diterpenoid metabolite of *Penicillium crustosum* has been a putative causal agent of a well-illustrated human toxicosis (Lewis et al., 2005).

2. Patterns of biosynthesis in fungi

The diversity of fungal secondary metabolite biosynthesis involves several of the amino acid components in protein synthesis, and notably all three of the essential aromatic amino acids (Mantle, 1987). Secondary products of fungi and plants, which incorporate tyrosine (**8**) or phenylalanine (**9**), commonly contain the whole carbon skeleton of the precursor, as may usually be obvious in structural diagrams. However, whereas those from tryptophan frequently conform generally to this apparent principle (e.g. the fumitremorgins and verruculogen (**10**), others are seen only to incorporate the indolic moiety. The latter are amply illustrated by several indole-diterpenoid fungal metabolites (e.g. penitrems, paxilline (**11**) and janthitrems from free-living *Penicillium* sp., and paspalines and lolitrems from 'ergot fungi' [*C. paspali* and *Neotyphodium lolii*]) parasitic on or endophytic in grasses. As indicated above, lolitrems became of economic importance as the long-elusive cause of the unique ryegrass staggers neuropathy in agricultural ruminants. However, since the fungal endophyte also expresses other beneficial properties, recent research has focused on genetic manipulation to prevent biosynthesis of lolitrem B (**3**) in the fungus (Saikia et al., 2008). Understanding the source of biosynthetic components of lolitrems is helpful in targeting genomes of enzymes of its pathways.

Whereas biosynthesis of the diterpenoid moiety of these compounds is well understood to arise *via* classical polyisoprenoid principles, tryptophan seemed to be the only source of the indole, although the mechanism for substituting the folded geranylgeranyl pyrophosphate to form the aliphatic side chain of the indole moiety of tryptophan (**6**) remained speculative. That the direct source of the indole is tryptophan (**6**) was inferred from several biosynthetic studies on fungi producing penitrem A (**2**) (de Jesus et al., 1883; Laws and Mantle, 1985, 1989), paxilline (**11**) (Laws and Mantle, 1989), and pennigritrem (**12**) (Penn et al., 1992), during which microgram amounts of tryptophan (**6**), ^{14}C-labelled uniformly to high specific activity only in the aromatic ring, were given to fermentations during the early part of the idiophase. This probe was available economically from Amersham International at that time only as residue from a custom synthesis; commissioning of another custom synthesis for extended studies would have been beyond university resources. However, the indole-diterpenoids that were studied all became radiolabelled as shown in autoradiograms of TLC plates, but by quite low percentage incorporation of the added ^{14}C (range 0.16–5%). Where the fungus also produced another metabolite in which the whole tryptophan molecule was indisputably incorporated (e.g. roquefortine (**13**) in *P. crustosum*), the percentage incorporation into roquefortine (**13**) was greater than that into penitrem A (**2**). The disparity was attributed partly to roquefortine (**13**) having the greater rate of synthesis during the period following addition of the probe, and partly to the greater difficulty of delivering an exogenous radiolabelled probe to travel efficiently to the intracellular sites of penitrem A (**2**) biosynthesis, that seemed to be located preferentially in *Penicillium* fructifications (Laws and Mantle, 1989).



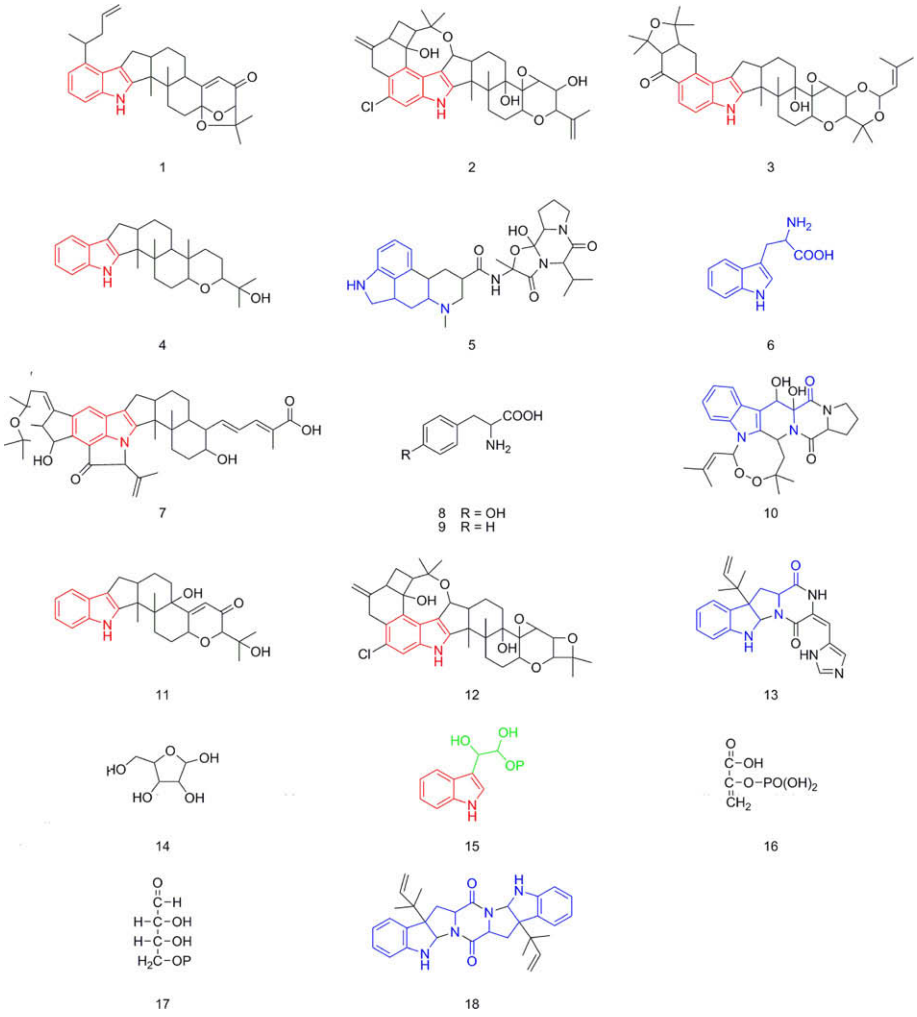
Scheme 1. Colour-coded graphic illustrating the basis of the current debate about the biosynthetic source of the indole moiety (red) of indole-diterpenoids from tryptophan or its immediate biosynthetic precursor indole-3-glycerol phosphate.

3. Biosynthesis of nodulisporic acid as a putative model for a common pathway to the indole moiety of indole-diterpenoids

Subsequent discovery of nodulisporic acid A (**7**) as a product of yet another endophytic fungus, and the industrial interest evoked by its insecticidal activity, justified comprehensive study of its biosynthesis in the Merck laboratories (Byrne et al., 2002). By use of ^{13}C-labelled putative precursors and NMR spectroscopy, biosynthesis of the isoprenoid regions was elucidated beyond doubt and the role of ribose (**14**) as the source of two carbons in the pyrrolic ring of the indole was elegantly demonstrated. Thus the role of at least the early steps in tryptophan (**6**) biosynthesis was confirmed. However, which intermediate in, or end-product of, tryptophan (**6**) biosynthesis served as the precursor for the indole moiety became an intriguing topic for speculation. In the event, it was deduced that indole-3-glycerol-phosphate (**15**), the penultimate compound in the tryptophan (**6**) biosynthetic pathway, was the key branch point for supplying the indole moiety to nodulisporic acid (**7**). In further discussion, wider application of this principle across fungal indole-diterpenoids was proposed. Adoption of the proposal with respect to lolitrems was explicit in a comprehensive review (Scott et al., 2004) and in a presentation by Scott at the 2006 International Mycological Congress in Cairns, expanded as Saikia et al. (2008). The publication from the Merck authors had rightly noted that previous biosynthetic studies with ^{14}C-labeled tryptophan lacked the specificity and robustness of ^{13}C NMR methodology (that was unfortunately beyond the experimental resources available for the work in the 1980s). They concluded that the low percentage incorporation values obtained during the ^{14}C studies represented scrambling of label *via* degradative processes in intermediary metabolism.

The conclusions from the nodulisporic acid (**7**) study have been adopted more widely (Scott et al., 2004; Saikia et al., 2008) and the topic of indole-diterpenoids has justified a review of their chemistry and bioactivity in which the indole-3-glycerol phosphate precursor is shown schematically (Sings and Singh, 2003). It now seems important to consider the experimental protocol used, and to find that it may not have been optimal for the purpose of determining the source of indole in indole-diterpenoids.

Mycelium had been taken after 5–6 days in a production stage batch fermentation (started with a 2.5% (v/v) inoculum of seed stage culture), when nodulisporic acid (**7**) production had reached 20–30 μg/ml. Mycelium was well-washed in a neutral non-nutritional buffer (MOPS) and resuspended in 20 mM MOPS buffer supplemented with glycerol (4% v/v). Label was added and the incubation shaken for a further 3–4 days. Total nodulisporic acid (**7**) was extracted from whole culture, but yield was not recorded, nor had there been measurement of its content of the washed cells used at the outset of the labelling experiment. Thus the mycelium used came from a stage late in idiophase, and presumably after exogenous nitrogen sources were exhausted. Efficient washing of mycelia would have ensured reliance only on endogenous sources and these would have been minimal since relatively abundant secondary productivity had already occurred in these cells. Adding a supplement of glycerol to the non-nutritive MOPS buffer would



have allowed carbon pathways to operate, but description of the mycelia as just in a 'resting stage' seems to be somewhat euphemistic. Rather, a descriptor of 'nitrogen-starved' could also be applied.

In these circumstances, it is somewhat surprising that the Merck authors did not use the sequential natural dynamics of batch fermentation with *Nodulisporium* and select the optimal rate of nodulisporic acid (**7**) biosynthesis as its idiophase commenced. Presumably all this information would have been available 'in house'. The problem with adding small amounts of tryptophan (**6**), as a biosynthetic probe, to N-starved mycelium is that the tryptophan (**6**) may primarily be used as a nitrogen source for primary metabolism.

Although no experimental details were given, the authors state that they were unable to label nodulisporic acid (**7**) with [indole-^{14}C]-tryptophan (**6**). This potentially makes an unfortunate starting point for subsequent discussion about the origin of the indole in nodulisporic acid (**7**) because, if the protocol had been similar to that described for the ^{13}C experiments, the experimental circumstances of restricted nutrition of cells derived from late idiophase in a batch fermentation would have been atypical. Similarly, failure to obtain positive data is not the best basis on which to build an alternative hypothesis unless the reason(s) for failure are understood. Whereas a genetic basis for aspects of diterpene biosynthesis has been perceived (Scott et al., 2004; Saikia et al., 2008) it is still premature to speculate concerning molecular mechanisms for incorporating the indole moiety into indole-diterpenoids. However, application of molecular methodology will become complementary in experimental resolution of the present debate. Indeed, since a feature of fungal secondary biosynthetic pathways is a less rigid reliance on particular enzymes than occurs in primary metabolic pathways, excision of either of the two similar aliphatic side-chains of indole-3-glycerol phosphate (**15**) and tryptophan (**6**) might be a flexible option open to fungi, allowing less dependence on metabolic status.

The proposal that the former positive ^{14}C findings from South African and UK studies could have arisen *via* metabolic scrambling of the aromatic ring of tryptophan (**6**) into degradative pathways (e.g. the kynurenine pathway) may be easy to suggest, but the feasible routes of degradative intermediary metabolism need to be scrutinised. It appears that other than very tortuous routes leading to the first precursors of tryptophan (**6**) (phospho-enol-pyruvate (**16**) and erythrose-4-phosphate (**17**)) do not occur (Matthews and van Holde, 1990). This authoritative text was also cited by Byrne et al. (2002). Further, degradative pathways may not have readily operated in the context of the highly anabolic metabolism during experimental ^{14}C probing of penitrem biosynthesis, and in the concurrently studied biosynthesis of roquefortine (**13**) or penigritrem (**12**) which more efficiently utilised the added tryptophan (**6**) radiolabelled in the aromatic ring. Similarly, easy routes

for tryptophan catabolites into isoprenoid pathways are also obscure.

It is proposed that additional experiments are necessary, designed to explore biosynthesis of several indole-diterpenoids during the natural dynamics of batch fermentation in shaken culture and using ^{13}C-labelled tryptophan. A fermentation system for studying the indole source in lolitrem biosynthesis in the context of its tryptophan-derived co-metabolite ergovaline (**5**) would be attractive, but is probably not yet available in sufficient product yield. However, several model systems are available in addition to that for nodulisporic acid (**7**) in the particular *Nodulisporium* sp., namely paxilline (**11**) alone in *P. paxilli* (Ibba et al., 1987), penitrem A (**2**) and pennigritrem (**12**) together in *P. nigricans* (Penn et al., 1992), penitrem A (**2**) and nigrifortine (**18**) together in *P. nigricans* (Laws and Mantle, 1985; Mantle et al., 1984), and janthitrems alone in *P. janthinellum* (Penn et al., 1993). Experiments conducted with ^{13}C and ^{14}C in parallel could also aid clarification of tryptophan's role as an indole source. Whereas in former experiments on penitrem A (**2**) radiolabelled tryptophan was delivered into the medium, it is now clear that application in surface droplets to stationary cultures can enable very efficient delivery of amino acid probes to biosynthetic sites in fungal mats (Miljkovic et al., 2008).

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References

Byrne, K.M., Smith, S.K., Ondeyka, J.G., 2002. Biosynthesis of nodulisporic A: precursor studies. J. Am. Chem. Soc. 124, 7055–7060.

Cole, R.J., Dorner, J.W., Lansden, J.A., Cox, R.H., Pape, C., Cunfer, B., Nicholson, S.S., Bedell, D.M., 1977. Paspalum staggers: isolation and identification of tremorgenic metabolites from sclerotia of *Claviceps paspali*. J. Agric. Food Chem. 25, 1197–1201.

Cole, R.J., Dorner, J.W., Cox, R.H., Raymond, L.W., 1983. Two classes of alkaloid mycotoxins produced by *Penicillium crustosum* thom isolated from contaminated beer. J. Agric. Food Chem. 31, 655–657.

de Jesus, A.E., Steyn, P.S., Van Heerden, F.R., Vleggaar, R.J., Wessels, P.L., 1883. Tremorgenic mycotoxins from *Penicillium crustosum*: biosynthesis of penitrem A. J. Chem. Soc., Perkin Trans. 1, 1863–1868.

Di Menna, M.E., Mantle, P.G., 1978. The role of Penicillia in ryegrass staggers. Res. Vet. Sci. 24, 347–351.

Fletcher, L.R., Harvey, I.C., 1981. An association of a *Lolium* endophyte with ryegrass staggers. NZ Vet. J. 29, 185–186.

Gallagher, R.T., Clardy, J., Wilson, B.J., 1980. Aflatrem, a tremorgenic toxin from *Aspergillus flavus*. Tetrahedron Lett. 21, 239–242.

Gallagher, R.T., White, E.P., Mortimer, P.H., 1981. Ryegrass staggers: isolation of potent neurotoxin lolitrem A and lolitrem B from staggers-producing pastures. NZ Vet. J. 29, 189–190.

Gallagher, R.T., Hawkes, A.D., Steyn, P.S., Vleggaar, R., 1984. Tremorgenic neurotoxins from perennial ryegress causing ryegrass staggers disorder of livestock: structure elucidation of lolitrem B. J. Chem. Soc. Chem. Commun., 614–616.

Hensens, O.D., Ondeyka, J.G., Dombrowski, A.W., Ostlind, D.A., Zink, K.L., 1999. Isolation and structure of nodulisporic acid A_1 and A_2, novel insecticides from a *Nodulisporium* sp. Tetrahedron Lett. 40, 5455–5458.

Ibba, M., Taylor, S.J.C., Weedon, C.M., Mantle, P.G., 1987. Submerged fermentation of *Penicillium paxilli* biosynthesizing paxilline, a process inhibited by calcium-induced sporulation. J. Gen. Microbiol. 133, 3109–3119.

Laws, I., Mantle, P.G., 1985. Nigrifortine, a diketopiperazine metabolite of *Penicillium nigricans*. Phytochemistry 24, 1395–1397.

Laws, I., Mantle, P.G., 1989. Experimental constraints in the study of the biosynthesis of indole alkaloids. J. Gen. Microbiol. 135, 2679–2692.

Lewis, P.R., Donoghue, M.B., Hocking, A.D., Cook, L., Granger, L.V., 2005. Tremor syndrome associated with a fungal toxin: sequelae of food contamination. Med. J. Aust. 182, 582–584.

Mantle, P.G., Mortimer, P.H., White, E.P., 1978. Mycotoxic tremorgens of *Claviceps paspali* and *Penicillium cyclopium*: a comparative study of effects on sheep and cattle in relation to natural staggers syndromes. Res. Vet. Sci. 24, 29–56.

Mantle, P.G., Laws, I., Tan, M.J.C., Tizard, M., 1984. A novel process for the production of penitrem mycotoxins by submerged fermentation of *Penicillium nigricans*. J. Gen. Microbiol. 130, 1293–1298.

Mantle, P.G., 1987. Secondary metabolites of *Penicillium* and *Acremonium*. In: Peberdy, J.F. (Ed.), Biotechnology Handbooks 1, In *Penicillium* and *Acremonium*. Plenum, New York, pp. 161–243.

Matthews, C.K., van Holde, K.E., 1990. Biochemistry. The Benjamin/Cummings Publishing Co., Inc., Redwood City, CA (pp. 717–719; 726).

Miljkovic, A., Collinson, L.M., Mantle, P.G., 2008. A radiochemical technique with potential for revealing novel fungal metabolites according to expression of biosynthetic activities. Mycol. Res. 112, 271–276.

Penn, J., Biddle, J.R., Mantle, P.G., Bilton, J.N., Sheppard, R.N., 1992. Pennigritrem, a naturally-occurring penitrem A analogue with novel cyclisation in the diterpenoid moiety. J. Chem. Soc., Perkin Trans. 1, 23–26.

Penn, J., Swift, R., Wigley, L.J., Mantle, P.G., Bilton, J.N., Sheppard, R.N., 1993. Janthitrems B and C, two principal indole-diterpenoids produced by *Penicillium janthinellum*. Phytochemistry 32, 1431–1434.

Richard, J.L., Bacchetti, P., Arp, L.H., 1981. Moldy walnut toxicosis in dogs, caused by the mycotoxin, penitrem A. Mycopathologia 76, 55–58.

Saikia, S., Nicholson, M.J., Young, C., Parker, E.J., Scott, B., 2008. The genetic basis for indole-diterpenoid chemical diversity in filamentous fungi. Mycol. Res. 112, 184–199.

Scott, D.B., Jameson, G.B., Parker, E.J., 2004. Isoprenoids: gene disorders and chemical puzzles. In: Tkacz, J.S., Lange, L. (Eds.), Advances in Fungal Biotechnology for Industry, Agriculture and Medicine. Springer, pp. 163–193.

Sings, H., Singh, S., 2003. Tremorgenic and non-tremorgenic 2,3-fused indole diterpenoids. In: Cordell, G.A. (Ed.), The Alkaloids: Chemistry and Biology, vol. 60. Elsevier Inc., pp. 51–163.

Wilson, B.J., Wilson, C.H., 1964. Toxins from *Aspergillus flavus*: production on food materials of a substance causing tremors in mice. Science 144, 177–178.

Wilson, B.J., Wilson, C.H., Hayes, A.W., 1968. Tremorgenic toxin from *Penicillium cyclopium* grown on food materials. Nature 220, 77–78.