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PAXILLINE BIOSYNTHESIS BY *ACREMONIUM LOLIAE*; A STEP TOWARDS DEFINING THE ORIGIN OF LOLITREM NEUROTOXINS

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Key Word Index—*Acremonium loliae*; Hyphomycetes; paxilline; lolitrem B; biosynthesis; tremorgenic mycotoxins; neurotoxin.

Abstract—Paxilline has been identified as a metabolite of the ryegrass endophyte *Acremonium loliae* in submerged culture and as a component, with lolitrem B, of the seed of endophyte-infected ryegrass. A role for paxilline as a biosynthetic precursor of lolitrem B is discussed.

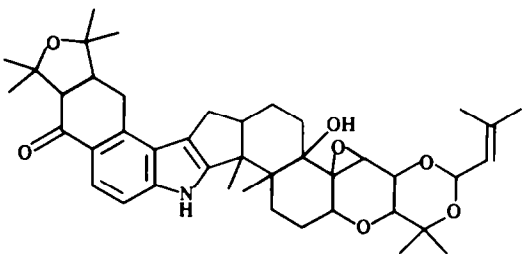
INTRODUCTION

Ryegrass staggers is a nervous disorder, notably of sheep and cattle. It is prevalent in parts of New Zealand in dry summer months when large numbers of animals become incoordinate when forced to move. The disorder also occurs in Australia, England and in other parts of the world where perennial ryegrass is a dominant pasture species. Ryegrass staggers is important principally since it imposes constraints on stock management. Mortality in severely incoordinate animals may occur, e.g. by drowning. After many years of searching for a cause, tremorgenic metabolites of some common mould fungi were proposed as causal agents [1], partly on the grounds of experimentally eliciting similar symptomatology and the fungi being readily accessible in pasture. The case for invoking tremorgenic mycotoxins has been subsequently reviewed [2]. In 1981 a novel potent neurotoxin, lolitrem B (1) (together with related lolitrems), was isolated from toxic perennial ryegrass [3] and closely correlated with the incidence of an endophytic fungus in this grass [4]. The endophyte has since been described as *Acremonium loliae* [5]. The structure of lolitrem B [6] is analogous to that of several indole-isoprenoid metabolites of fungi, many of which similarly have tremorgenic properties, notably aflatrem [7], penitrems [8], paspalinines [9], janthitrems [10] and paxilline (2) [11].

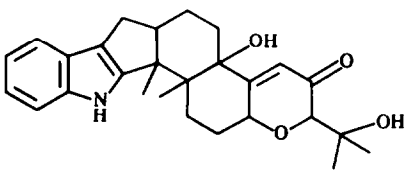
It is now generally accepted that lolitrem neurotoxins, occurring particularly in the leaf sheaths of perennial ryegrass at the rate of several ppm [12], cause ryegrass staggers, but the biosynthetic origin of the molecule (whether it is a product of the fungus, the plant, or of both) is obscure. So are the factors which determine the remarkable fact that one of the most important pasture grasses in temperate regions may become lethal. Eradication of the endophyte by reseeding with endophyte-free seed might be a desirable long-term strategy were it not that the fungus has been shown to increase the vigour of the grass and to confer significant protection against insect pests [13]. An insect antifeedant, peramine, produced by *A. loliae* has recently been characterized [14]. Thus, though known for about half a century but being regarded as inconsequential, the ryegrass endophyte has recently assumed considerable significance. Nevertheless, apparently efforts to demonstrate lolitrem biosynthesis by *A. loliae* in pure culture have been consistently unsuccessful.

RESULTS AND DISCUSSION

In the course of exploring potential to biosynthesize indole-isoprenoid metabolites, an isolate of *A. loliae*, obtained from New Zealand ryegrass, was found to produce paxilline; 4 mg were purified from 200 ml of the fermented culture. The metabolite was identical to authentic paxilline produced by, for the first time also in submerged fermentation, the particular strain of *Penicillium paxilli* that is otherwise the only organism to yield paxilline [15, 16]. For example, the *A. loliae* metabolite cochromatographed with paxilline in TLC and



1 (Lolitrem B)



2 (Paxilline)

HPLC and gave the same electron impact mass spectrum [m/z 435 (M^+) and important fragment ions m/z 417, 377, 359, 344, 182 (base peak), 58, 43]. The ^1H NMR spectrum was also identical to that of authentic paxilline and was in accord with the published spectrum [17].

Paxilline has also been identified, occurring at *ca* 3 ppm together with a similar amount of lolitrem B [12, 18], in endophyte-infected English ryegrass seed.

The unexpected finding of paxilline as a principal secondary metabolite of *A. loliae* in axenic culture, in the absence of any evidence that the organism was producing lolitrem B, is the first evidence that this ryegrass endophyte can biosynthesize indole-isoprenoid neurotoxin. The striking similarity of paxilline to the corresponding indole-isoprenoid moiety of lolitrem B, the biosynthesis of which can be predicted to involve tryptophan and a diterpene by analogy with paspalinines and penitrems, implies that *A. loliae* has the enzymes to elaborate the indole-diterpenoid moiety of lolitrems. The coincidence of paxilline and lolitrem B in endophyte-infected ryegrass further suggests that paxilline may be a normal intermediate in lolitrem biosynthesis. That the aromatic substitution of paxilline with two isoprenes is also achievable by the endophytic fungus may be implied by analogy with penitrems and janthitrems, which are metabolites of *Penicillium* spp. *A. loliae* is taxonomically close to *Cephalosporium acremonium*, which is also metabolically close to *Penicillium chrysogenum* since both elaborate penicillins. Thus, there are phylogenetic grounds for at least not being surprised at some similarity in secondary metabolism between *A. loliae* and *Penicillium* spp. [16]. Further, if the *A. loliae* metabolite peramine is derived essentially as a cyclic anhydride of proline and arginine [14], such biosynthesis is matched in the penicillia by several amino acid-derived diketopiperazines [16].

Lolitrems are apparently unique amongst the indole-isoprenoid tremorgens in the additional isoprene attached to two oxygen atoms. The disposition of the oxygen substituents in this region of the paxilline molecule is favourable for such an addition of an isoprene but, taken together with the aromatic substitution with isoprenes, whether the putative transformations of paxilline to give lolitrems are mediated directly by the fungus, or also involve the host plant, remains to be elucidated. Nevertheless, the present findings concerning paxilline biosynthesis in *A. loliae* offer a reasonable basis for ascribing biosynthesis of the structurally related lolitrem B to the endophyte.

EXPERIMENTAL

Penicillium paxilli Bain. (ATCC 26601) was grown for 10 days in 500 ml flasks containing 100 ml Czapek Dox broth supplemented with yeast extract (0.5%). Flasks were incubated at 27° on a rotary shaker (200 rpm, 10 cm eccentric throw). Freeze-dried culture was extracted with Me_2CO overnight and the extract chromatographed through a column of silica gel using $CHCl_3$–Me_2CO (19:1) to give crude paxilline. Paxilline was purified to homogeneity by reversed phase HPLC through an ODS column (250 × 10 mm) using MeOH–H_2O (6:1), flow rate 4 ml/min and UV detection at 230 nm. This authentic sample of paxilline, retention volume 22 ml, was crystallized from MeOH.

Acremonium loliae was grown for 34 days in 500 ml flasks containing 200 ml medium consisting of glucose (2%), peptone (0.5%), yeast extract (0.25%) and pectin (1%). Flasks were incubated at 23° on a rotary shaker. Freeze-dried culture was extracted with Me_2CO overnight and the extract chromatographed, with authentic paxilline as a reference (R_f 0.3) over silica gel using $CHCl_3$–Me_2CO (19:1). The *A. loliae* component which cochromatographed with authentic paxilline was eluted from the silica gel with $CHCl_3$–propan-2-ol (10:1) and analysed chromatographically (TLC and HPLC) and by mass and ^1H NMR spectroscopy. Analytical TLC [silica gel GF_{254} (Camlab)] required development (× 3) in $CHCl_3$–Me_2CO (19:1) to achieve optimum resolution at R_f 0.6. HPLC through a Waters Radial-PAK μ Bondapak NH_2 cartridge (10 cm × 8 mm) used CH_2Cl_2–propan-2-ol (100:1) at a flow rate of 4 ml/min and UV detection at 281 nm. Paxilline had a retention volume of 8 ml and a quantitative assay was possible over the range 0–2 μg.

Lolium perenne L. spikelets (1 g) containing mature seed from a permanent English pasture were analysed for lolitrem B by a method based on that previously described [18]. Extraction in 15 ml $CHCl_3$–MeOH (2:1) and Sep-pak preparation of lolitrem B for HPLC was identical to [18]. HPLC used a Waters Radial-PAK silica cartridge and a mobile phase of CH_2Cl_2–CH_3CN (15:1). A Perkin–Elmer 1000 M fluorimeter with 268 nm narrow band pass interference excitation filter and 450 nm wide band pass emission filter required injection of the extract of 30 mg seed in 100 μl $CHCl_3$. At a flow rate of 2 ml/min the retention volume of a reference sample of lolitrem B was 12 ml.

The same seed extract, omitting Sep-pak treatment, was also analysed directly for paxilline in the HPLC system described above for analysis of *A. loliae* extract, except that propan-2-ol was omitted from the mobile phase. One tenth of the extract was evaporated to dryness and the residue taken up and injected in 20 μl $CHCl_3$.

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