The susceptibility of MB to enzymatic digestion is not presently known, but could be an important concern. Several peptides, such as prolactin[15], TRF[16], in addition to milk- and food-derived morphinomimetic peptides[23], may be absorbed from the stomach within minutes, permitting physiologically active forms to reach the blood stream with minimal proteolytic degradation. Other factors in milk may contribute to the stability of peptides as well.

In view of the known pharmacological and physiological effects of amphibian bombesin in mammals[9-12, 17, 18], MB may also function as a 'nutrient hormone'[13] and, by analogy with amphibian bombesin, may be capable of influencing smooth muscle contractility, release of gastrointestinal hormones and prostaglandins[14, 24] and regulation of tissue growth. It may also be capable of acting synergistically to potentiate the effects of other hormones such as the GRP-stimulated release of ACTH by CRF[25].

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The potent tremorgenic neurotoxins lolitrem B and aflatrem: A comparison of the tremor response in mice

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Ruakura Animal Research Station, Ministry of Agriculture and Fisheries, Private Bag, Hamilton (New Zealand), 9 September 1985

Summary. Tremor dose-response curves were determined for mice dosed with the ryegrass neurotoxin lolitrem B, and the tremorgenic mycotoxin aflatrem. A family of characteristic curves was revealed for each tremorgen, with lolitrem B eliciting a sustained tremor response persisting for over 24 h.
Key words. Tremorgenic neurotoxins; indoles; lolitrem **B**; aflatrem; mice; tremor; dose-response, ryegrass staggers.

The lolitrems are remarkable tremorgenic neurotoxins which have recently been isolated from perennial ryegrass (*Lolium perenne* L.) and ryegrass seed[1-3]. When injected i.p. into laboratory mice, these lipophilic neurotoxins induce a neurotoxic syndrome characterized by sustained pronounced tremors[1, 4]. The structure of the major lolitrem neurotoxin, lolitrem B, of mol.wt 685 and formula $C_{42}H_{55}NO_7$, has been determined and shown to be a complex substituted indole (**1**)[5]. The lolitrem neurotoxins are the prime suspect causative agents of ryegrass staggers, a dramatic nervous disorder of sheep, cattle, horses and deer grazing ryegrass-dominant pastures[6-10]. Animals affected by this disorder exhibit tremors, severe incoordination and hypersensitivity to external stimuli, yet there is a consistent lack of observable specific lesions in even severely affected animals, and such intoxicated animals usually show eventual complete recovery and return to normality[3, 8-11]. Since ryegrass staggers occurs frequently in New Zealand, Australia and the United Kingdom, there is considerable interest in the causative neurotoxins[6-14]. A mouse bioassay[1, 4], based on the sustained tremorgenic response induced in mice by the neurotoxins, was originally used to screen and estimate the relative neurotoxicity of pasture samples taken from pastures on which livestock had developed ryegrass staggers. The bioassay was also utilized in the initial isolation and purification work on the lolitrems[1]. Very recently a rapid, sensitive and quantitative method based on high performance liquid chromatography (HPLC) with fluorescence detection[3], has been developed for lolitrem B estimation in pasture samples and ryegrass plant components.

The unique neurotoxicology of the lolitrems (viz. sustained tremor, absence of primary lesions, rapid return to normality of severely intoxicated animals) warrants investigation. The purpose of the present investigation is to provide some quantitative data on the potency of pure lolitrem B by establishing a dose-response relationship in mice and comparing its action with a well-known tremorgenic neurotoxin, aflatrem (**2**)[15-18], produced by the ubiquitous fungus *Aspergillus flavus*.

Materials and methods. An appropriate volume of a stock solution of lolitrem B or aflatrem (available from the authors' re-

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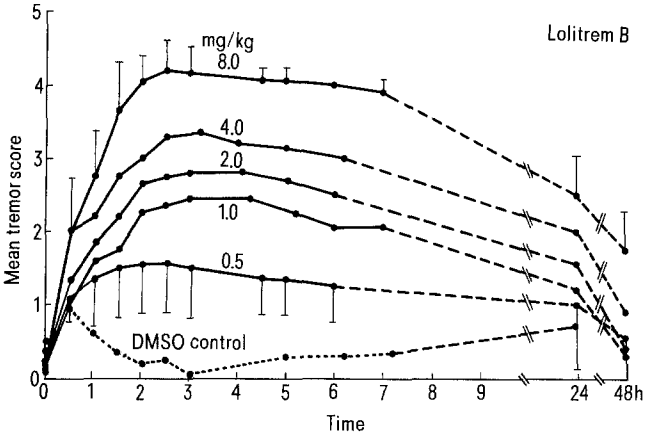


Figure 1. Mean tremor score elicited in groups of mice dosed with lolitrem B (0.5, 1.0, 2.0, 4.0, 8.0 mg/kg, i.p.) versus time post injection. Each point represents the mean ± SD (n = 10 mice).

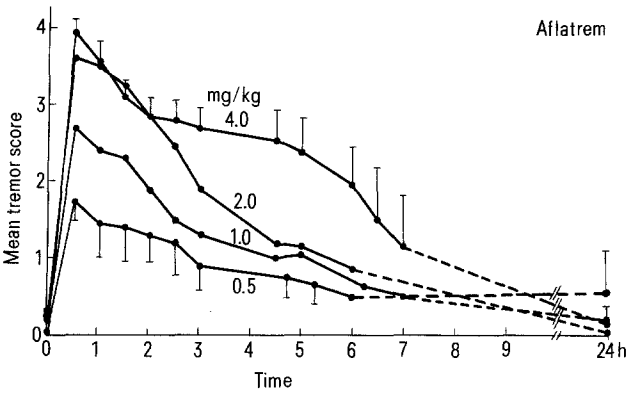


Figure 2. Mean tremor score elicited in groups of mice dosed with aflatrem (0.5, 1.0, 2.0, 4.0 mg/kg, i.p.) versus time post injection. Each point represents the mean ± SD (n = 10 mice).

search programme) in dichloromethane: acetonitrile (80: 20, v/v) was transferred to a glass vial and evaporated under N_2. The resulting solid toxin residue was redissolved in 1.26 ml dimethyl sulphoxide (DMSO) with the aid of a vortex mixer, then 0.14 ml H_2O was added and mixed to give a toxin solution in 90% DMSO. The toxin was injected i.p. in 0.1 ml of 90% DMSO carrier into a 6–8-week-old white mouse (25 ± 5 g b.wt). Ten mice were injected at each dose rate. Lolitrem B was dosed at 5 levels (0.5, 1.0, 2.0, 4.0, 8.0 mg/kg). Aflatrem was dosed at 4 levels (0.5, 1.0, 2.0, 4.0 mg/kg). Ten mice were also dosed with 0.1 ml of 90% DMSO/25 g mouse as an injection carrier control. All mice used were female, CF No. 1 (Wallaceville Animal Research Centre, Upper Hutt, New Zealand) and were maintained on supplemented lucerne pellets and water ad libitum.

Mice were observed at 30-min-intervals for 3 h post injection then at approximately hourly intervals for at least 6 h post injection, for a neurotoxic response. A further observation was made at 24 h post injection. Whole body tremor was assessed using a visual scoring system with a rating scale of 0–5 (see table)[4].

Results and discussion. Injection of mice with lolitrem B or aflatrem elicited a neurotoxic syndrome in which several neurobehavioural signs were apparent. The signs most frequently observed included: incoordination (especially manifested in an uncoordinated gait of the moving animal), hyperactivity, hypersensitivity (particularly sensitivity to sound and tactile stimuli), and tremor. Whole body tremor was the most notable and consistent tremor mode elicited, and the neurotoxic response in this and other investigations was assessed on the extent of the whole body tremor observed. A visual rating scale (table) was adopted for the tremor assessment[4]. Such visual rating scales have been effectively used in the past for tremor assessment in rats and mice dosed with tremorgenic agents, such as the indole tremorgen harmine[19]. With these neurotoxins, the whole body tremor observed at lower doses usually occurs in 'bursts' of pronounced periods of tremor activity, interspersed by periods of quiescence, or inactivity, and then a further tremor burst. Observation of the number of tremor bursts, and whether or not resting tremor was present, allowed an easy and repeatable distinction to be made between the scores on the rating scale.

Visual rating scale for tremor assessment

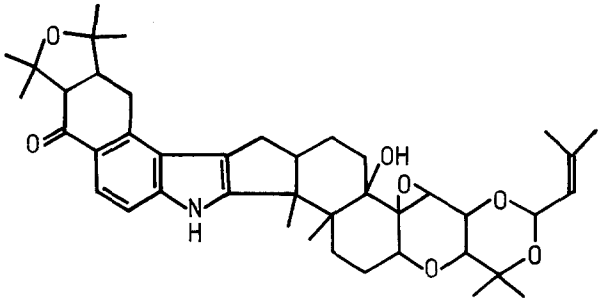
0	No tremor, animal behaviour normal.
1	No resting tremor. A short-duration, low-intensity single-burst whole body tremor elicited by exercise/handling.
2	No resting tremor. Several moderate-intensity whole body tremor burst elicited on exercise/handling.
3	Spontaneous, continuous low-intensity resting tremor may be present. Repeated moderate to severe intensity tremor bursts elicited on exercise/handling.
4	Pronounced, protracted, spontaneous resting tremor. Movement, exercise, or handling may induce convulsive episodes in addition to severe tremor.
5	Severe spontaneous tremor, usually accompanied by convulsive episodes, and eventually culminating in death.

All dose rates of lolitrem B (0.5–8.0 mg/kg) used gave a detectable tremorgenic response at 30 min post injection (p.i.), with a maximal response being reached in 2–3 h p.i., see figure 1. This maximal response plateaued between approximately 2.5–5 h p.i. then slowly declined in intensity. At 6 h p.i. the response was still $\geqslant 80\%$ of the maximum attained, and even at 24 h p.i. it was substantially above control levels. By 48 h p.i. there was a barely detectable response. The mice were kept for a week after injection: at this time, feeding and behavior for all mice appeared normal.

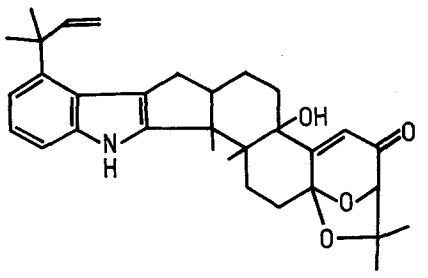
All dose rates of aflatrem gave a sharp, maximal response at 30 min p.i., with a subsequent fall-off in tremorgenic response which was much more rapid than that observed for lolitrem B, see figure 2. AT 6 h p.i. the response was only ca 50% of the maximum for the 4 mg/kg dose, whilst for the lower doses of aflatrem the response was marginal. At 24 h p.i., there was a barely detectable response. After one week p.i. feeding and behavior for all mice appeared normal.

The sustained duration of action of tremor elicited by dosing with tremorgenic neurotoxins such as lolitrem B is a remarkable phenomenon. Most compounds that induce tremors produce only a transient tremor: in 1956 Everett et al.[20] reported that of 10,000 drugs and compounds screened, less than 10 had the ability to produce sustained tremor. The finding by Everett et al. that the drug tremorine evoked sustained tremor in a number of animal species, was a major pharmacological discovery at that time. Tremorine, a potent tremorgenic agent, and its even more potent metabolic derivative oxotremorine, subsequently found utilization as an important tool by pharmacologists and neurobiologists for investigation into the phenomenon of tremor and several CNS disorders[21]. These synthetic neurotoxic compounds produced a sustained tremor response in dosed animals lasting several hours: the lolitrems, and some tremorgenic mycotoxins have a greater tremor potency (duration of effect) than this[1,8,22]. Since the discovery of aflatrem, the first tremorgenic mycotoxin isolated, more than 20 such mycotoxins have been discovered[10,22,23].

The dose level of 4.0 mg lolitrem B/kg elicited a near-maximal tremor response, from which all mice eventually returned to



(1)



(2)

normal behaviour. This reversible nature of the tremorgenic syndrome is consistent with the production of a reversible biochemical lesion affecting neurotransmission[10]. The highly lipophilic, neutral properties of the lolitrems together with their medium molecular weight range are consistent with the hypothesis[24] that the toxins (and/or their active metabolites) are capable of gaining access to the central nervous system where they exert their primary effects. The possession of an indole nucleus by the neurotoxins is believed to be an important feature contributing to their unique neurotoxicology.

The use of aqueous dimethyl sulphoxide (DMSO) in the present investigation as a neurotoxin carrier for dosing by i.p. administration to mice, was advantageous. The glass pestle and grinding tube procedure previously used for dispersion of lolitrem-containing ryegrass extracts in aqu. NaCl/Tween 80 was convenient in that the prepared extracts contained lipid material which emulsified with the dispersing agent (Tween 80) to form a uniform preparation suitable for injection purposes. However an inherent problem in the latter procedure for the quantitative sample preparation and i.p. injection of lolitrem B and aflatrem in their pure forms, was the difficulty in forming uniform and stable suspensions of the pure toxins in the predominantly aqueous carrier. This problem was overcome when lolitrem B and aflatrem were found to be completely soluble in a 90% solution of DMSO in H_2O, which proved to be a convenient and suitable carrier for i.p. dosing. The dose rate of DMSO used as carrier was 4.0 g/kg b.wt in this investigation, which is well below the LD_{50} level of 20.1 g/kg determined by Caujolle et al.[25] for mice. Although the i.p. injection of DMSO in mice is reported to have some toxic effects[26], the level used in the present investigation elicited a minimal tremor response – see DMSO control, in figure 1. Also, in earlier investigations (unpublished results), i.p. injections of detergent suspensions of lolitrem B gave very similar results (potency and time-course-of-action) to the aqueous DMSO toxin solutions reported on here.

In conclusion, a comparison has been made of the tremor-producing potency in white mice of lolitrem B and the tremorgenic mycotoxin aflatrem. Dose-response curves were determined for a range of dose levels of both lolitrem B (0.5–8.0 mg/kg) and aflatrem (0.5–4.0 mg/kg). A family of characteristic curves was revealed for each tremorgen, with lolitrem B showing an unusually sustained tremor response. The tremorgenic potency and reversible neurotoxicity of these lipophilic indole-containing molecules is of importance in view of their involvement in natural tremorgenic disorders, and of much pharmacological interest.

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