Tremorgenic Neurotoxins from Perennial Ryegrass causing Ryegrass Staggers Disorder of Livestock: Structure Elucidation of Lolitrem B

Rex T. Gallagher,*[a] Allan D. Hawkes,[a] Pieter S. Steyn,[b] and Robert Vleggaar*[b]

[a] *Ruakura Animal Research Station, Ministry of Agriculture and Fisheries, Private Bag, Hamilton, New Zealand*
[b] *National Chemical Research Laboratory, Council for Scientific and Industrial Research, P.O. Box 395, Pretoria 0001, Republic of South Africa*

The lolitrems, tremorgenic neurotoxins isolated from perennial ryegrass, are implicated in ryegrass staggers disorder in livestock; the assignment of structure (**1**) to the major neurotoxin, lolitrem B, is based on its spectroscopic properties, particularly a detailed study of its high-field ^{1}H and ^{13}C n.m.r. spectra, as well as chemical evidence.

Ryegrass staggers is a nervous disorder of sheep, cattle, horses, and deer grazing perennial ryegrass (*Lolium perenne* L.) dominant pastures.[1,2] The disorder, characterized by severe inco-ordination and hypersensitivity to external stimuli, is of considerable importance to agriculture in New Zealand and Australia, with occasional outbreaks also being recorded in other countries, including the United Kingdom.[3,4] A remarkable feature of the disorder is the consistent lack of observable specific lesions in severely affected animals and the eventual complete recovery and return to normality of such intoxicated animals.[2,4] Extensive investigations into the cause of ryegrass staggers recently led to the isolation and purifica-

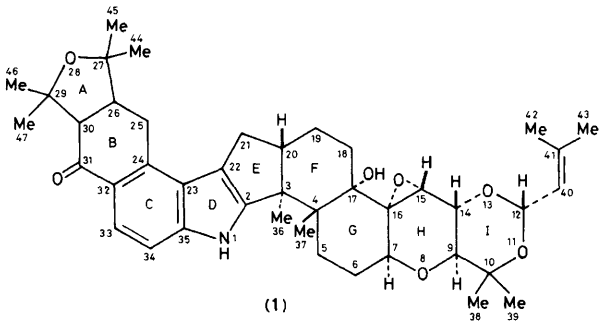
tion of four potent neurotoxins, named lolitrems A, B, C, and D, from toxic ryegrass and ryegrass seed.[5,6] We now report on the structure (**1**) of the major neurotoxin, lolitrem B, based on its spectroscopic properties and particularly on a detailed study of its high-field ^1H and ^{13}C n.m.r. spectra. The tryptophan-mevalonate derived structure of this toxin is remarkable in that it contains twelve chiral centres in a linear array of ten contiguous fused rings.

Lolitrem B (**1**), m.p. 303—304 °C, was analysed as $C_{42}H_{55}NO_7$ by high resolution mass measurement of the molecular ion, *m/z* 685.3974.[5] An abundant ion at *m/z* 348 arises through cleavage of the C-3–C-4 and C-18–C-19 bonds with transfer of a hydrogen atom to the indole-containing fragment. A similar diagnostic fragmentation is observed for the related tremorgens aflatrem,[7] paspalinine,[7] and penitrem A.[8]

The i.r. spectrum exhibited absorptions at ν_{max} (KCl) 3530, 3480, and 3315 cm^{-1}, assigned to OH and NH groups and a carbonyl absorption band at 1664 cm^{-1}.

Absorptions at λ_{max} (MeOH) 290 (ε 6 700) and 267 nm (26 800) in the u.v. spectrum of lolitrem B suggested the presence of a 2,3-disubstituted indole nucleus with a carbonyl group in conjugation with the aromatic ring. This supposition was verified by sodium borohydride reduction of lolitrem B which gave a mixture of two diastereoisomeric alcohols (M^+, 687 and *m/z* 350) each with absorptions at λ_{max} 232 and 285 nm, characteristic of the indole chromophore present in *e.g.* penitrem B.[8]

A feature of the 500.13 MHz ^1H n.m.r. spectrum of lolitrem B was the three-proton signals at δ_H 1.717 (d, *J* 1.3 Hz), 1.711 (d, *J* 1.3 Hz), 1.515, 1.370, 1.301, 1.276, 1.276, 1.266, 1.240, and 1.134 assigned to the protons of ten tertiary methyl groups. The remainder of the resonances in the ^1H n.m.r. spectrum exhibited extensive fine structure. First-order analyses of these multiplets yielded the values of the proton chemical shifts and proton–proton coupling constants. From the values of the coupling constants, as corroborated by



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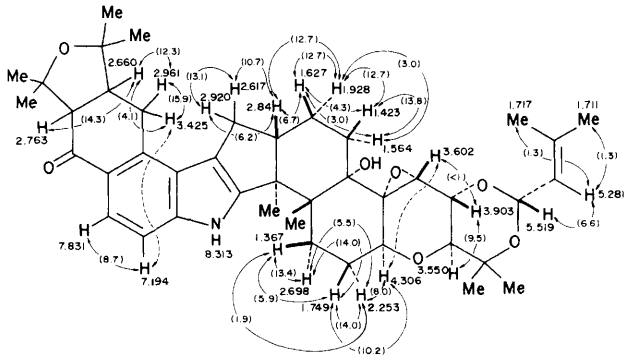

Figure 1. The ^1H chemical shifts and coupling constants (Hz) for lolitrem B. The (^1H,^1H) connectivity pattern as indicated was determined by homonuclear decoupling experiments. The broken lines show cases where effects were observed during decoupling experiments, although no splittings were measurable.

Table 1. 125.76 MHz ^{13}C N.m.r. data for lolitrem B (**1**).[a]

Carbon atom	δ_C/p.p.m.[b]	^1J(CH)/Hz	Δδ[c]	Carbon atom	δ_C/p.p.m.[b]	^1J(CH)/Hz	Δδ[c]
2	152.77(s)		−0.162	26	49.87(d)	128	
3	50.69(s)			27	79.26(s)		
4	42.36(s)			29	79.93(s)		
5	27.38(t)			30	59.93(d)	126.7	
6	27.98(t)			31	196.51(s)		
7	71.45(d)	149		32	136.97(s)		
9	71.21(d)	147		33	120.23(d)	162.3	
10	74.70(s)			34	110.42(d)	161.0	
12	92.66(d)	160.6		35	142.00(d)		−0.146
14	71.11(d)	139.6		36	15.91(q)[d]		
15	61.13(d)	181.2		37	16.59(q)[d]		
16	67.73(s)			38	18.87(q)[d]		
17	78.04(s)		−0.113	39	25.01(q)[d]		
18	30.26(t)			40	121.96(d)	158.3	
19	20.49(t)			41	139.54(s)		
20	50.08(d)	122		42	25.65(q)		
21	29.16(t)			43	18.63(q)		
22	118.58(s)			44	25.10(q)[d]		
23	125.96(s)			45	28.28(q)[d]		
24	123.89(s)			46	29.31(q)[d]		
25	28.28(t)			47	30.63(q)[d]		

[a] Recorded on a Bruker WM-500 spectrometer. [b] Relative to Me_4Si, solvent $CDCl_3$. [c] Deuterium isotope shifts (in p.p.m.) observed on addition of H_2O–D_2O (1 : 1). [d] May be interchanged.

extensive $^1H\{^1H\}$ homonuclear decoupling experiments, the (^1H,^1H) connectivity pattern of the lolitrem B molecule could be constituted (see Figure 1).

The ^{13}C n.m.r. data for lolitrem B (**1**) as collated in Table 1 were obtained from broad-band proton-decoupled and single frequency nuclear Overhauser effect (n.O.e.) spectra. The multiplicities of the different ^{13}C resonances were determined by generating the proton-decoupled CH, CH_2, and CH_3 subspectra using the DEPT pulse sequence.[9] The signals of all the proton-bearing carbon atoms were correlated in turn with specific proton resonances in a two-dimensional (^{13}C,^1H) shift correlation experiment.[10] In the assignment of the different ^{13}C n.m.r. resonances use was made of the two- and three-bond (C,H) connectivity pattern as determined by heteronuclear $^{13}C\{^1H\}$ selective population inversion (SPI)[11] experiments, and the reported ^{13}C n.m.r. chemical shifts and (C,H) coupling constants of related compounds *e.g.* the penitrems.[8,12]

The location of the carbonyl group at C-31 rather than C-25 follows from the chemical shift of the C-33 proton (δ_H 7.831) in lolitrem B. In contrast the corresponding proton in penitrem D and E, which both lack the *peri* carbonyl moiety, resonates at δ_H 6.702.[12] Furthermore, when the C-33 proton transitions were irradiated in a SPI experiment, the signal at δ_C 196.51 p.p.m., assigned to C-31 was affected. The presence of the 2-methylprop-1-enyl moiety at C-12 instead of C-10 is evident from the chemical shift values of the 12-H (δ_H 5.519, d, J 6.6 Hz) and C-12 (δ_C 92.66 p.p.m.) resonances.

The relative configuration of lolitrem B was deduced from the proton–proton coupling constants as well as the proton–proton n.O.e.s.[13] A comparison of the proton chemical shifts and coupling constants for the protons of the E—I fragment in lolitrem B with those of the corresponding protons in the penitrems[8,12] leads to the assumption that the relative and absolute configuration at C-3, C-4, C-7, C-15, C-16, C-17, and C-20 is the same as for the penitrems. The n.O.e. observed between the C-9 proton and 7-H, but not 14-H, shows that rings H and I are *trans*-fused with 9-H *cis* to 7-H. The *trans* configuration of 9-H and 14-H is based on the fact that no n.O.e. is observed between these two protons as well as the vicinal (H,H) coupling constant of 9.5 Hz. The C-14 chiral centre in lolitrem B must therefore have the *R*-configuration whereas the corresponding centre in the penitrems has the enantiomeric configuration. This deduction was confirmed by the vicinal (H,H) coupling constant of <1 Hz for the C-14 and C-15 protons, which is indicative of a dihedral angle of *ca.* 90°. The appreciable n.O.e. observed between H-14 and H-12 established the configuration at C-12.

Although the *trans*-fusion of rings A and B follows from the vicinal (H,H) coupling constant of 14.3 Hz for the C-26 and C-30 protons, the relative and absolute configuration of these two chiral centres remains unknown.

The ^1H n.m.r. spectrum of a minor metabolite, lolitrem C ($C_{42}H_{57}NO_7$, M_r 687) indicates that the C-40–C-41 double bond present in lolitrem B has been reduced.

It is evident that the lolitrems are related to the known tremorgenic mycotoxins, *viz.* aflatrem,[7] the penitrems,[8,12] and janthitrems[14] in terms of structure, biogenesis, and biological effects.[15] The structural differences in rings A and B of the lolitrems, penitrems, and janthitrems are due to different isoprenylations which in turn lead to the unique ring structures. In the case of the lolitrems, an additional mevalonate unit leads to the formation of ring I. The above findings are of cardinal importance in terms of recent reports that an *Acremonium* species, an endophytic fungus which infects ryegrass, is associated with the production of the neurotoxins which cause ryegrass staggers.[16]

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