Corynetoxins, Causative Agents of Annual Ryegrass Toxicity; Their Identification as Tunicamycin Group Antibiotics

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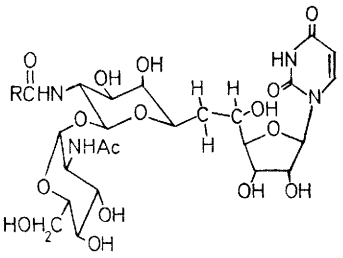
The corynetoxins, formed in galled seeds of *Lolium rigidum* (annual ryegrass) occupied by *Corynebacterium rathayi* and responsible for annual ryegrass toxicity, are identified as new members of the tunicamycin group of antibiotics.

The toxicity of annual ryegrass (*Lolium rigidum*) to grazing animals is associated with infestation of the seedheads by a nematode, *Anguina agrostis*,[1] and a bacterium, *Corynebacterium rathayi*.[2] The toxicity resides in the galled seedheads occupied by the bacterium.[3,4] This lethal infestation of pastures is of major and increasing importance to the sheep industry in South and Western Australia[5] and has recently appeared in South Africa.[6] A family of toxins, named corynetoxins, capable of reproducing the disease in sheep and experimental animals, was recently isolated from the bacterial galls.[7] We report here the identification of the corynetoxins as new members of the tunicamycin group of antibiotics (**1**) (Figure 1) produced by species of *Streptomyces*.[8]

The tunicamycins (**1**) have a common uracil–tunicamine–*N*-acetylglucosamine moiety with different fatty acids linked *via* amide bonds to the tunicamine amino-group.[8–10] The corynetoxins (**2**) (Figure 1) differ in the fatty acids which have slightly longer chains, a β-hydroxy series in addition to the $\alpha\beta$-unsaturated and saturated series, and anteiso forms as well as the iso and normal forms found in the tunicamycins.[11]

The corynetoxins identified and their order of elution from a reversed-phase h.p.l.c. column are shown in Figure 2, where individual toxins are specified by a term indicating the acid series, carbon number, and methyl branching. The main components are corynetoxins H17a (β-hydroxy, C_{17}, anteiso) and U17a ($\alpha\beta$-unsaturated, C_{17}, anteiso). They have molecular weights of 876 and 858 (Table 1) (by field desorption and fast atom bombardment mass spectrometry) in accord with the proposed structures, and on hydrolysis with dilute HCl yield *N*-acetylglucosamine, tunicaminyl-uracil, and fatty acids identified as 3-hydroxy-14-methylhexadecanoic acid and 14-methylhexadec-2-enoic acid respectively.

N-Acetylglucosamine from hydrolysis of corynetoxins H17a and U17a was identified as its pentatrimethylsilyl (TMS) derivative (M^+, m/z 539) by gas chromatography–mass spectrometry (g.c–m.s.). Further hydrolysis gave glucosamine



Tunicamycins (**1**)[10]
Acids R—CO_2H[a]
$\alpha\beta$-Unsaturated: n-C_{14}, n-C_{16}; i-C_{13}—i-C_{17}; C_{15},[b] C_{17}[b]
Saturated: i-C_{15}

Corynetoxins (**2**)
Acids R–CO_2H[a]

β-Hydroxy	$\alpha\beta$-Unsaturated	Saturated
i-C_{16}, i-C_{18}	i-C_{16}—i-C_{18}	i-C_{16}, i-C_{18}
a-C_{17}, a-C_{19}	a-C_{17}, a-C_{19}	a-C_{15}, a-C_{17}
		a-C_{19}

Figure 1. Structures of tunicamycins and corynetoxins [a] n = normal, i = iso, a = anteiso. [b] Unspecified chain branching.

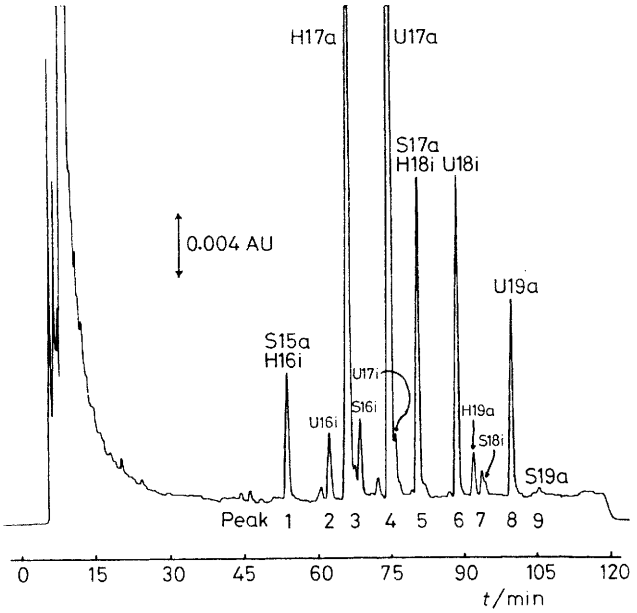


Figure 2. Reversed-phase h.p.l.c. of corynetoxins from galled seedheads of annual ryegrass (*Lolium rigidum*) (bonded ODS phase, 5 μm particle size, methanol–water gradient, 55 °C, u.v. detection). The main components are identified by the acid series [H (hydroxy), U (unsaturated), or S (saturated)], carbon number, and chain termination [n (normal), i (iso), or a (anteiso)].

which was identical (g.c.–m.s. and electrophoresis) with an authentic sample.

Tunicaminyl-uracil hydrochloride was isolated from the acid hydrolysis of a sample of mixed corynetoxins. Its ^{1}H n.m.r. spectrum (270 MHz) comprised the complete spectrum of an authentic sample supplied by Dr. R. L. Hamill, with identical chemical shifts and coupling constants, together with additional signals due to impurities. The two samples had the same mobilities on electrophoresis and t.l.c. which were also used to demonstrate the formation of tunicaminyl-uracil from corynetoxins H17a and U17a. Further hydrolysis of tunicaminyl-uracil from corynetoxins H17a and U17a yielded uracil which was identified by g.c.–m.s. as its TMS derivative (M^+, m/z 256) and by electrophoresis and u.v. comparison with authentic uracil.

Table 1. Molecular weights from field-desorption mass spectrometry.

H.p.l.c. peak	$[M+Na]^+$ ion	M	Corynetoxin[a]
3	899	876	H17a
4	881	858	U17a
5	883	860	S17a
5	913	890	H18i
6	895	872	U18i
7	927	904	H19a
8	909	886	U19a

[a] See Figure 2.

The identity of the fatty acids of corynetoxins H17a and U17a was established by g.c.–m.s. of the methyl esters of the acids released on hydrolysis and by the ^{1}H n.m.r. spectra of the intact toxins. The C_{17}, monounsaturated, methyl ester (M^+, m/z 282) from corynetoxin U17a showed an intense fragment ion at m/z 113 characteristic of $\alpha\beta$-unsaturated esters.[12] After reduction to the saturated methyl ester (M^+, m/z 284) it showed the g.c. retention time (co-chromatography) and characteristic m.s. fragmentation of authentic methyl 14-methylhexadecanoate. The methyl ester of the C_{17} hydroxy-fatty acid from corynetoxin H17a ($M^+ - H_2O$, m/z 282) and its *O*-trimethylsilyl derivative ($M^+ - CH_3$, m/z 357) showed intense fragment ions at m/z 103 and 175 respectively which are characteristic of 3-hydroxy-fatty acid methyl esters and their TMS derivatives.[13]

The ^{1}H n.m.r. spectrum of corynetoxin H17a showed uracil doublets at δ 7.90 and 5.77; three anomeric sugar CH doublets at δ 5.91, 5.03, and 4.67; partially overlapping CHOH and CHOR multiplets at δ 4.22—3.34; a doublet due to CHOH–CH_2–CONH at δ 2.31; a singlet due to $COCH_3$ at δ 1.98; two multiplets at δ 2.10 and 1.57, due to non-equivalent CH_2 protons in CHOH–CH_2–CHOR; a $[CH_2]_n$ peak at δ 1.30; a CH_2 multiplet at δ 1.15; a methyl doublet at δ 0.85; and a triplet at δ 0.86, due to –$CH(CH)_3$–CH_2CH_3. The spectrum of U17a is closely similar, but has a sextet at δ 6.80 and a doublet at δ 5.99 due to *trans*-$\alpha\beta$ unsaturated CH_2–CH=CH–CONH, and the CHOH–CH_2–CONH peak is absent. With the expected differences in methyl peaks, the spectrum of U17a closely matches the spectrum of a sample of tunicamycin C1 (which contains an iso, $\alpha\beta$-unsaturated, C_{16} acid) also supplied by Dr. Hamill. All other defined multiplets common to H17a, U17a, and tunicamycin C1 show closely similar coupling constants and chemical shifts. The similarity in data for the anomeric CH groups shows that the stereochemistry at the anomeric linkages is the same in the two sets of compounds. This is confirmed also by ^{13}C n.m.r. data. The lack of aldehydic properties shows that the linkage of tunicaminyl-uracil and *N*-acetylglucosamine in the corynetoxins occurs through the anomeric carbon atoms as in the tunicamycins.

Other individual corynetoxins have been purified or partially purified by a combination of reversed-phase and silica h.p.l.c., the latter separating the β-hydroxy series from the $\alpha\beta$-unsaturated and saturated series. Molecular weight measurements were made on the major constituents (Table 1) and each has been hydrolysed in acid with identification of *N*-acetyl-glucosamine and the fatty acid (g.c.–m.s.) and tunicaminyl-uracil (t.l.c.). Thus the corynetoxins differ only in the fatty acid components. Further evidence includes the reversed-phase h.p.l.c. elution pattern (Figure 2), in which the order reflects differences in fatty acid polarity and chain length and the quantitative conversion (confirmed by h.p.l.c.) of the unsaturated acid corynetoxins into the saturated series by selective hydrogenation of the fatty acid double bond.

Co-chromatography (h.p.l.c.) of mixed corynetoxins with an authentic sample of tunicamycins gives precise coincidence

of peaks where expected. At least five components appear to be common. They are S15a, U16i, U17a, and U17i. The structures of U16i, and U17i (tunicamycins VII and X), were established by Ito *et al.*;[10] those of the other three are indicated by the present work.

The tunicamycins are potent inhibitors of protein glycosylation in a wide variety of cell types[14–16] and have antibiotic activity. Preliminary studies confirm that the corynetoxins share both properties. This sheds considerable light on the mechanisms underlying the pathological effects of annual ryegrass toxicity and suggests roles for the toxins in bacteria-occupied seed galls of *L. rigidum*. These implications will be discussed elsewhere.

We thank Dr. R. L. Hamill, Lilly Research Laboratories, for samples of tunicamycin C1 and tunicaminyl-uracil and VG Analytical Ltd. for fast atom bombardment spectra.

Received, 12th October 1981; Com. 1200

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