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E/Z-Thesinine-*O*-4′-α-rhamnoside, pyrrolizidine conjugates produced by grasses (Poaceae)

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ABSTRACT

Based on direct infusion mass spectrometry we identified a novel alkaloid as a major component of perennial ryegrass (*Lolium perenne*). Initial mass spectral data suggested it to be a pyrrolizidine conjugate. As this class of alkaloids has not been described before from grasses, we isolated it to elucidate its structure. The isolated alkaloid proved to be a mixture of two stereoisomers. The structures of the two compounds as determined by 1D and 2D NMR spectroscopy, were *E*-thesinine-*O*-4′-α-rhamnoside (**1**) and *Z*-thesinine-*O*-4′-α-rhamnoside (**2**). These identifications were supported by the characterisation by GC-MS and optical rotation of (+)-isoretronecanol as the necine base released on alkaline hydrolysis of these alkaloids. **1** and **2** together with the aglycone and a hexoside were also detected in tall fescue (*Festuca arundinacea*). This is the first report of pyrrolizidine alkaloids produced by grasses (Poaceae).

1. Introduction

Perennial ryegrass (*Lolium perenne*) and tall fescue (*Festuca arundinacea)* are important pasture grasses providing the main feed for grazing livestock in many countries e.g. USA, Australia, and New Zealand. These two grasses have been the subject of intense chemical investigation because of their toxic effects on grazing mammals, particularly in relation to the syndromes commonly known as ryegrass staggers and fescue foot. This led to the discovery of a number of grass alkaloids such as perloline, a diazaphenanthrene alkaloid (Grimmett and Waters, 1943; Jeffreys, 1964; Bush and Jeffreys, 1975), which however were not responsible for the toxicoses. During the 1970s these livestock toxicoses were associated with the occurrence of endophytic fungi in *L. perenne* and *F. arundinacea*, and attention moved to fungal alkaloids, leading to the discovery of the fungal metabolites lolitrem B (Gallagher et al., 1984), and ergovaline (Lyons et al., 1986), primarily responsible for the animal toxicity. Since then almost all recent chemical attention regarding pasture grasses has been focused on fungal secondary metabolism and no further alkaloids produced by the host grasses have been described.

Recent metabolomic investigations of the *L. perenne* endophyte symbiosis have revealed the presence of novel grass alkaloids. Based on direct infusion mass spectrometry (Koulman et al., 2007) an ion for an unknown alkaloid of *m/z* 434 was observed to predominate in many *L. perenne* extracts independent of the presence of endophytic fungi. This predominant ion showed some variation between tissues and treatments (Fig. 1), but was not identified amongst those most significantly affected by the presence of endophyte in the grass (Cao et al., 2008.) and therefore the elucidation of its chemical identity was not an initial priority. However, because it was a major constituent of the *L. perenne* metabolome, we decided that it was prudent to characterise it.

2. Results and discussion

Infusion of an extract into the ESI source of the mass spectrometer and collision-induced fragmentation and high resolution Fourier Transformation mass spectrometry of the major ion (*m/z* 434) provided information about the parent species and neutral loss and product ion fragments. The $[MH]^+$ ion (*m/z* 434.2175 = $C_{23}H_{32}NO_7$, calculated mass 434.2173) underwent a neutral loss of 146.0576 ($C_6H_{10}O_4$; calculated mass 146.0579) leaving a product fragment of *m/z* 288.1597 ($C_{17}H_{22}NO_3^+$; calculated mass 288.1594) that underwent a further neutral loss of 146.0370 ($C_9H_6O_2$; calculated mass 146.0368), leaving a product fragment of *m/z* 142.1227 ($C_8H_{16}NO^+$; calculated mass 142.1226). We determined the UV λ_{max} to be 305 nm, by LCMSPDA which together with the neutral loss fragment of molecular formula $C_9H_6O_2$ suggested that the chromophore was a coumaryl moiety and the rest of the molecule did not have any chromophores. Based on the accurate mass we assigned the first neutral loss fragment as deriving from a putative

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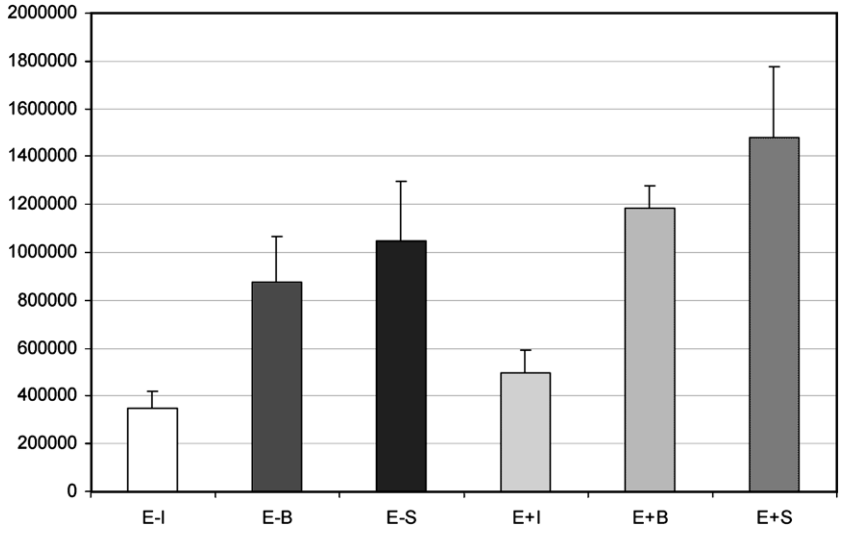

Fig. 1. Variation in intensities of the ion of *m*/z 434 in DIMS of extracts of different tissues of *Lolium perenne* uninfected (E − I: uninfected immature tissue, E − B; uninfected blade tissue, E − S: uninfected sheath tissue) or infected with *Neotyphodium lolii* (Lp19) endophyte (E + I: infected immature tissue, E + B; infected blade tissue, E + S: infected sheath tissue) (n = 3, mean ± stdev).

rhamnoside, and the remaining product ion fragment as a putative alkaloid. The LCMS also showed that there were two partially resolved compounds with the same fragmentation pattern and similar UV spectrum suggesting that we were dealing with a mixture of two stereo isomers (**1**/**2**). The aglycone part of the molecule corresponded in its molecular formula to thesinine, a saturated pyrrolizidine conjugate. However as the reported occurrence of pyrrolizidine alkaloids has thus far been limited to a select number of plant families (Reimann et al., 2004), none of which are related to grasses, we considered more evidence of the chemical structure was required.

The mixture of stereoisomers was extracted from *L. perenne* herbage (110 g dry wt) with MeOH using a Soxhlet extractor. The extract was made alkaline using NH_3 and partitioned between water and CH_2Cl_2. The organic phase was concentrated and subjected to two silica column separations. This resulted in 62 mg of yellow–brown oil, which comprised the two stereoisomers with other little interfering material based on LCMSPDA (λ 305 nm). This was subjected to NMR spectroscopy in solution in either a $CDCl_3$/d_4–MeOH mixture or D_2O.

The proton and carbon spectrum in the $CDCl_3$/d_4–MeOH mixture gave signals corresponding to those published for the aglycone part of thesinine-glucoside (Herrmann et al., 2002), confirming the presence of a thesinine conjugate. However the spectrum also showed clear evidence of a second distinct component. To determine the complete structure of both compounds, we chose D_2O as NMR solvent because it allowed us to determine the long-range coupling between the coumaryl part and the alkaloid part in the HMBC spectrum, which failed in the $CDCl_3$/d_4–MeOH mixture (Herrmann et al., 2002). The NMR data for the two components of the mixture are reported in Tables 1 and 2. Based on the coupling constants in the ^{1}H NMR spectrum we were able to determine that the purified fraction comprised a pair of stereoisomers differing in configuration around the coumaryl double bond. Thus the olefinic protons of the coumaryl group showed couplings consistent with either a *Z* (*J* = 12.3 Hz) or *E* (*J* = 16.1 Hz) configuration, and these were observed in a near 1 to 1 ratio. The 3′/5′ aromatic ring proton signals for the two stereoisomeric coumaryl moieties were overlaid, but two distinct sets of 2′/6′ proton doublets (*J* = ca. 8.5 Hz) were observed (Tables 1 and 2). An apparent isolated doublet at 5.54 ppm was assigned as the anomeric proton of the sugar group. Careful examination of the spectrum

Table 1
The ^{1}H (500.1 MHz) and ^{13}C (125.7 MHz) NMR data of *E*-thesinine-*O*-4′-α-rhamnoside (**1**) (in D_2O)

C	^{13}C	^{1}H	*J* (HZ)	COSY	H2BC	HMBC
1	39.2	2.79	m	9/8	2,8,9	2a/b, 9a/b 7a 6a
2	25.4	a1.9	ol	1,3a/b	1,3	ol
		b2.1	ol	3b		
3	54.0	a3.26	m	2a/3b	2	2a/b, 5a
		b3.45	m	2a/b		
5	56.2	a2.93	m	6a	6	3a/b,6a,7a
		b3.75	m	6a		
6	25.4	a1.83	ol	5a/b,7a/b	5,7	ol
		b2.15	ol	3b,5a, 7b		
7	25.3	a1.82	ol	8	6,8	ol
		b2.05	ol	8		
8	69.1	4.236	ol	1/7	1,7	1,2b,3a/b,5a,7a
9	63.2	a4.23	ol	1	1	1,2a,5a
		b4.32	dd(10.6,7.3)			
1′	128.5					7′,8′
2/6′	130.1	7.54	d(8.6)	3/5′	3/5′	3/5′,7′
3/5′	117.1	7.1	ol	2/6′	2/6′	2/6′
4′	157.4					1″,3/5′,2/6′
7′	145.7	7.62	d(16)	8′	8′	
8′	115.4	6.38	d(16)	7′	7′	7′
9′	169.0					9,7′,8′
1″	97.9	5.54	d(1.3)	2″	2″	
2″	69.8	4.09	bs	1″,3″	1″, 3″	4″,1″
3″	70.0	3.92	dt(9.7,2.6)	2″,4″	2″, 4″	ol
4″	71.9	3.45	t(9.7)	3″,5″	3″, 5″	2″,3″,6″
5″	69.5	3.67	dt(6.4,9.7)	4″,6″	4″, 5″	6″
6″	16.6	1.14	d(1.3)	5″	6″	4″

and further evidence from the 2D spectra revealed that this signal comprised two doublets each with a very small coupling constant (*J* = 1.3 Hz), assigned to the anomeric protons of the two stereoisomers. Through a selective TOCSY experiment at 5.54 ppm with 150 ms mixing time we were able to obtain a proton spectrum of the sugar group confirming that this was a rhamnoside type sugar with a methyl group signal at 16.6 ppm in the ^{13}C spectrum. As the value of the coupling constant of H-1″ was less than 7 Hz, it was determined to be α-rhamnose (Agrawal, 1992). By overlaying the sugar signals with the complete proton spectra we chose a well-isolated multiplet at 2.646 ppm for a second selective TOCSY experiment. This gave a series of signals between 1.5 and 4.5 ppm accounting for all the protons of the pyrrolizidine part of

Table 2
The ^{1}H (500.1 MHz) and ^{13}C (125.7 MHz) NMR data of *Z*-thesinine-*O*-4′-α-rhamnoside (**2**) (in D_2O)

C	^{13}C	^{1}H	*J* (HZ)	COSY	H2BC	HMBC
1	38.8	2.64	m	9/2a	2,8,9	2a/b, 9a/b 7a 6a
2	25.4	1.80	m	1,3a/b	1,3	ol
		1.97	m	3b		
3	53.8	3.36	m	2a/3b	2	2a/b, 5a
		3.15	m	2a/b		
5	56.0	3.68	dt (5.3,11.0)	6a	6	3a/b,6a,7a
		2.86		6a		
6	25.4	1.75	m	5a/b,7a/b	5,7	ol
		2.01	m	3b,5a, 7b		
7	25.3	1.66	m	8	6,8	ol
		1.85	m	8		
8	69.0	4.03	m	1/7	1,7	1,2b,3a/b,5a,7a
9	63.2	4.13	dd (8.0,10.7)	1	1	1,2a,5a
		4.25	dd (6.1,1.2)			
1′	129.6					8′,7′
2/6′	130.9	7.41	d (8.5)	3/5′	3/5′	3/5′,7′
3/5′	116.6	7.1	ol	2/6′	2/6′	2/6′
4′	156.0					1″,3/5′,2/6′
7′	143.8	7.1	d (12.1)	8′	8′	
8′	118.0	5.92	d (12.3)	7′	7′	7′
9′	168.5					9,7′,8′
1″	97.6	5.55	d (1.3)	2″	2″	
2″	69.8	4.09	bs	1″, 3″	1″, 3″	4″, 1″
3″	70.0	3.92	dt (9.7,2.6)	2″, 4″	2″, 4″	ol
4″	71.9	3.45	t (9.7)	3″, 5″	3″, 5″	2″, 3″, 6″
5″	69.5	3.67	dt (6.4,9.7)	4″, 6″	4″, 5″	6″
6″	16.6	1.15	d (6.0)	5″	6″	4″

2. A third selective TOCSY experiment was performed at a free double doublet at 4.335 ppm, which yielded the proton signals of the pyrrolizidine part of **1**.

The structures of the three groups of both stereoisomers could then be assigned combining information from the DQF-COSY, HSQC, H2BC, HMBC and NOESY spectra. The spectra of the sugar moiety showed the least difference between the two stereo isomers. Only the anomeric signals were slightly separated and the methyl group gave only one signal in the carbon spectrum. However, in the proton spectrum it appeared as a triplet as a result of two overlapping doublets (*J* = 6.0 Hz). Based on the HMBC spectrum it was clear that the anomeric proton of the sugar is coupled to C4′ of the coumaryl group at 156 ppm (Table 2) and this linkage was further confirmed by a nOe between the anomeric proton of the rhamnose and the 2′/6′ protons of the aromatic ring in the NOESY spectrum. The carbonyl of the coumaryl unit (C9′) at 168.5 ppm is linked in the HMBC spectrum with the C9 protons at 4.13 and 4.25 ppm in the alkaloid moiety. The C9 carbon and its protons are linked to C1 at 39 ppm and the C1 proton. The ring structure of this part of the molecule could be unambiguously determined as a pyrrolizidine system by a heteronuclear 2-bond correlations (H2BC) spectrum. This is a relatively new 2D NMR spectroscopy method that allows two bond relationships between protons and proton-bearing carbons to be detected, independent of longer-range ^{1}H–^{13}C coupling constants (Nyberg et al., 2005). Thus the connectivities C1–C2–C3, C1–C8–C7–C6–C5 and C1–C9 could be established for both components (Tables 1 and 2). The COSY spectrum established that the pyrrolizidine moiety of both components had the same stereochemistry. In both cases a weak coupling was observed between C1-H and both C8-H and C2-Ha but a strong coupling between C1-H and C9-H. No coupling was observable between C1-H and C2-Hb. The NOESY spectrum shows a stronger nOe between C1-H and C8-H than between C1-H and C9-H. This established C1-H is *syn* to C8-H but *gauche* to the geminal C2 protons, and hence the C1–C9 bond is *trans* to the bridgehead C8-H bond in both components. This stereochemistry is that of isoretronecanol. Thus we have isoretronecanol (or enantiomer) linked through an ester bridge to *E* coumaryl-4′-*O*-α-rhamnoside (**1**) and *Z* coumaryl-4′-*O*-α-rhamnoside (**2**) (Fig. 2a), and the MS fragmentation pattern could be assigned as in Fig. 2b.

Confirmatory evidence of structures **1** and **2** including evidence for the absolute configuration as shown in Fig. 2 was provided by alkaline hydrolysis. Two *p*-coumaric acid 4-*O*–rhamnoside isomers were released, as identified by LCMSMS, together with the alkaloid base which was identified as isoretronecanol by GC-MS comparison with trachelanthamidine and isoretronecanol prepared from an alkaloid extract of a *Phalaenopsis* hybrid orchid by alkaline hydrolysis (Frölich et al., 2006) and as (+)-isoretronecanol by its positive optical rotation.

Thesinine, isolated from borage (*Borago officinalis*, Boragiaceae) (Dodson and Stermitz, 1986), has been characterised as isoretronecanol linked through an ester bridge to *E*-*p*-coumaric acid. Thesinine-4′-*O*-β-D-glucoside has also been isolated and characterised from borage (Herrmann et al., 2002). The presence of *Z*-thesinine or *Z*-thesinine glucoside in borage (or any other species) has not been reported.

The discovery of a pyrrolizidine alkaloid in grasses as a plant product is very remarkable. Pyrrolizidine alkaloids which have an ether-bridge between the C2 and C7, and a nitrogen C9-substituent are well-known from *F. arundinacea* where they occur as the products of endophytic fungi (*Neotyphodium* species) and there is evidence they have an important role as insect deterrents (Wilkinson et al., 2000). However pyrrolizidine alkaloids have been reported in only a few plant families, none closely related to grasses, and the biosynthesis of fungal and plant pyrrolizidines is quite different (Blankenship et al., 2005; Faulkner et al., 2006). Within the monocots, they are known from only one other family, the Orchidaceae, and in that case also only 1,2-saturated pyrrolizidine alkaloids have been reported (Frölich et al., 2006). Plant pyrrolizidine alkaloids as a class are infamous for their toxicity towards mammals and insects, but this toxicity is observed only for those pyrrolizidine alkaloids with C1–C2 unsaturation and a hydroxy group at C7 (Hartmann, 1999). As the grass pyrrolizidine alkaloid conjugates identified in this study have neither, their toxicity to mammals is likely to be limited.

Supporting evidence for this view was provided by a limited survey of several grass cultivars by LCMSMS which showed these alkaloids were also present in *L. perenne* cultivars Nui D, Impact and Samson as well as *F. arundinacea* cultivars Jesup and Kentucky 31. The LCMSMS analysis showed that most of the grass samples that contained **1** and **2**, also contained hexose conjugates of **1** and **2** (likely a glycoside), and *F. arundinacea* also accumulates the aglycone, thesinine. All these compounds gave a *m/z* 288 product ion fragment in the MS^2, which fragments further as described for **1**/**2**. We have sought ions in the LCMSMS chromatograms that might suggest the presence of 1,2-unsaturated version of isoretronecanol, either free, or conjugated to a coumaryl group and sugars (see material and methods, for selected masses), but thus far have not found any evidence for such species.

When we investigated grass plants of the same cultivar from the greenhouse we found wide variations in the level of **1**/**2**, and with variation in the ratios of **1** to **2** between 0.2 and 1 (data not shown). Clonal material grown in a climate room under carefully controlled conditions did not show such a large variation (see Fig. 1; Cao et al., 2008), suggesting that environmental factors have a strong influence on the accumulation of *E*/Z-thesinine-rhamnoside.

As these pyrrolizidines are abundant in commercial cultivars of grasses in wide use in agriculture it is unlikely that they cause any severe toxicity for mammals. It has been suggested that 1,2 saturated pyrrolizidines might have deterring effects on insect herbivores, but there is no direct evidence for this (Frölich et al., 2006). However, their common occurrence in commercial cultivars suggests that plant breeders may have inadvertently selected for

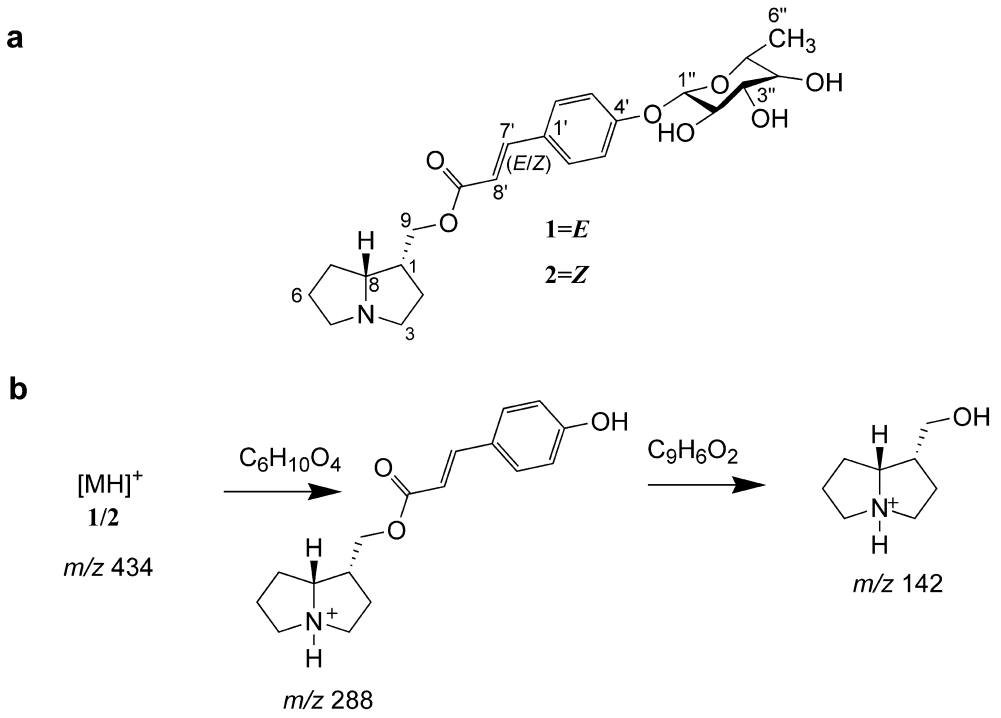

Fig. 2. (a) Structure of *E*/*Z*-thesinine-rhamnoside isolated from *Lolium perenne*. (b) Major pathway of fragmentation of protonated *E*/*Z*-thesinine-rhamnoside (*m*/*z* 434) in a linear ion trap using 35% relative collision energy.

their presence which implies they may have favourable effects on plant performance, for example, persistence. Further study is required to define the activity and role of these compounds.

3. Experimental

3.1. General procedures

Gas chromatography-mass spectrometry was carried out on a Shimadzu 2010 instrument (Shimadzu Corporation, Kyoto, Japan) fitted with a capillary column (DB1-MS, 30 m × 0.25 mm ID × 0.25 μm film thickness, J&W Scientific). Optical rotations were measured on a NPL Automatic Polarimeter 143D (Thorn Bendix Ltd., Nottingham, UK) equipped with an Hg lamp (589 nm filter) and a 1 dm cell.

3.2. ESIMS

Mass spectra were determined with a linear ion trap mass spectrometer (Thermo LTQ) using ESI in +ve mode. The spray voltage was 5.0 kV and the capillary temperature 275 °C. The flow rates of sheath gas, auxiliary gas, and sweep gas were set to 20, 5, and 10 (arbitrary units), respectively. Other parameters were optimized automatically by infusing peramine in H2O:MeCN:HCO_2H (95:5:0.1, v/v/v) at a flow rate of 200 μl min^{-1}. UV spectra were obtained using a Thermo Surveyor PDA detector (200–600 nm).

Accurate mass on the protonated compound and its fragments from CID in an ion trap was obtained on a Thermo Finnigan LTQ FT, which is a combination of a linear ion trap mass spectrometer and a Fourier transform ion cyclotron resonance mass spectrometer.

3.3. NMR

Nuclear magnetic resonance (NMR) spectra were recorded using a Bruker Avance 500 MHz spectrometer equipped with a probe optimized for inverse detection. Selective TOCSY experiments (mixing time 150–200 ms) were used to identify individual spin systems of glycoside and alkaloid groups. ^{1}H–^{13}C connectivities were from phase-sensitive heteronuclear single quantum coherence (HSQC) and heteronuclear multiple bond correlation (HMBC) experiments, and the ^{13}C chemical shifts of were confirmed by direct measurement of the ^{13}C spectrum. Double quantum filtered correlation spectroscopy (DQF-COSY) and nuclear Overhauser effect spectroscopy (NOESY) experiments (the latter with a mixing time of 1 s) were then used to order coupling networks within glycoside residues and to confirm some long-range HMBC connectivities. Standard instrument parameters were used throughout. Two-dimensional (2D) experiments were performed using the echo-antiecho method. Each spectrum contained 256 rows of 1024 points. Linear prediction followed by zero filling was applied to the indirect dimension giving a final size of 1024 × 1024 points. ^{1}H NMR spectra were referenced to residual HOD at δ4.70 ppm.

3.4. Plant material

Herbage of perennial ryegrass (*Lolium perenne* L.) Nui infected with *Epichloë festucae* Fl1 was used for the preparative isolation, and the other grass plants investigated were grown in individual pots in the greenhouse from seed lines obtained from Margot Forde Forage Germplasm Centre, AgResearch Grasslands, Palmerston North, New Zealand. Leaf material from a *Phalaenopsis* hybrid orchid was provided by Accent on Flowers, Palmerston North, New Zealand. Plant samples were freeze dried and milled and stored in a freezer (−20 °C) prior to extraction and isolation.

3.5. Extraction and isolation of compounds

Two batches of milled lyophilised herbage of perennial ryegrass (ca. 55 g each) were extracted with hexane in a Soxhlet until the pigmentation (chlorophyll plus lipids) was removed (ca. 4 h), followed by an extraction with MeOH for 5 h. The MeOH fraction

was concentrated to 100 ml under reduced pressure. About 50 ml of H_2O was added and the pH value was increased with 1% NH_4OH buffer to pH 10 and the extract was partitioned three times with 100 ml of $CHCl_3$. The chloroform fraction was concentrated and subjected to open CC using silica gel, collecting 25 ml fractions. Elution was carried out with a series of solvents of increasing polarity from 1 l of $CHCl_3$:Me_2CO:HCO_2H (60/40/0.1), 0.5 l of $CHCl_3$:Me_2CO:MeOH:HCO_2H (25/10/65/0.1), 0.5 l of $CHCl_3$:MeOH:HCO_2H (10/90/0.1) and ending with 0.5 l of Me_2CO:MeOH:HCO_2H (10/90/0.1). To clean the column afterwards, it was eluted with MeOH with 0.1% HCO_2H. The fractions containing the compound of interest (detected by LCMS) were combined, the pH adjusted to 10 with 1% NH_4OH buffer and the mixture partitioned for 3 times with 50 ml $CHCl_3$ to transfer the compound to the $CHCl_3$ phases. The $CHCl_3$ phases were combined, concentrated and subjected to open CC using silica gel, eluting with 1 l $CHCl_3$:MeOH (25/75) followed by 600 ml of MeOH with 0.1% HCO_2H. This yielded 62 mg of an enriched fraction.

3.6. Identification of the necine base

Isolated alkaloid (ca. 10 mg) was dissolved in aqueous NaOH (2.5%, 5 ml) and the mixture was allowed to stand at room temperature with stirring for 10 m. LCMSMS analysis of an aliquot of the reaction mixture with positive ESI detection as described below showed the disappearance of the parent compound (m/z 434) and with negative ESI detection the appearance of a pair of peaks (m/z 309) putatively *E* and *Z* *p*-coumaric acid 4-*O*–rhamnoside. *Iso*-PrOH (15 ml) was added to the mixture together with saturated aqueous NaCl (10 ml) to aid partitioning. The organic layer was separated, dried over anhydrous Na_2SO_4, and the *iso*-PrOH evaporated under reduced pressure to give isoretronecanol as an oily residue (1.1 mg). A necine base fraction was prepared for comparison by base hydrolysis of an alkaloid extract from a *Phalaenopsis* hybrid orchid, by an adaptation of the procedure of Frölich et al. (2006). Aliquots of isoretronecanol and the *Phalaenopsis* necine base fraction were dissolved in EtOAc and analysed by GC-MS under the conditions described by Frölich et al. (2006). Isoretronecanol was identified by comparison of the mass fragmentation pattern with reference data (Wiley and NIST libraries) and by co-elution with isoretronecanol and separation from trachelanthamidine from *Phalaenopsis* (Frölich et al., 2006).

3.7. Screening of grass extracts

Fresh plant material (between 100 and 200 mg fresh weight) was collected in screw cap vials. For the extraction 1.5 ml of MeOH:H_2O (1/1) and two ceramic beads were added to each vial. The vials were shaken vigorously in a BIO101/Savant FastPrep FP120 (Qbiogene, Carlsbad, CA, USA) at 4.0 ms^{-1} for 45 s, after which the tubes were rotated for 1 h at 30 rpm. After extraction each vial was centrifuged at 13,000 rpm and 900 μl of the supernatant was transferred to an HPLC vial. Analytes were eluted through a C18 Luna column (Phenomenex, Torrence, CA, USA) (150 × 2 mm, 5 μm) at a flow rate of 200 μl min^{-1} using a Thermo Finnigan Surveyor HPLC system with a solvent gradient (solvent A: H_2O 0.1% HCO_2H; B: MeCN 0.1% HCO_2H), starting with 3% B, 97% A for 5 min and then increasing to 23% B over 15 min followed by a column wash at 95% B. **1**/**2** were detected in SRM mode, selecting m/z 434.2 ± 2.5, 35% relative collision energy, and quantifying the m/z 288.2 fragment ion. Furthermore the MS was set to collect fragmentation data in data dependent manner using a parent mass list (m/z 140, 142, 156, 227, 286, 288, 382, 404, 432, 434, 444, 448, 450, 451, 561, 594, 596, 612) or otherwise the most intense ion.

3.7.1. E-Thesinine-O-4′-α-rhamnoside (**1**)

Yellow oil; UV λ_{max} MeCN/H_2O (log ε): 235, 305 nm (3.2). ^1H and ^{13}C NMR (500 MHz, D_2O) see Table 1. HRESIMS, m/z $[MH]^+$ 434.2175 (Calcd. for $C_{23}H_{32}NO_7$, 434.2173). ESIMS (positive ion mode; m/z, rel int (%)) 434.2 $[MH]^+$ ms^2 434.20 @ 35% CE: 288.2 (100), 147.2 (2.6), 142.2 (4.1), 124 (0.6), ms^3 434.20 @ 35% CE, 288.20 @ 35% CE: 147.0 (13.2), 142.12(17.3), 124.0 (100).

3.7.2. Z-Thesinine-O-4′-α-rhamnoside (**2**)

Yellow oil; UV λ_{max} MeCN/H_2O (log ε): 230, 290 nm (3.2). ^1H and ^{13}C NMR (500 MHz, D_2O) see Table 2. HRESIMS, m/z $[MH]^+$ 434.2175 (Calcd. for $C_{23}H_{32}NO_7$, 434.2173). ESIMS (positive ion mode; m/z, rel int (%)) 434.2 $[MH]^+$ ms^2 434.20 @ 35% CE: 288.2 (100), 147.2 (0.5), 142.2 (1.5), ms^3 434.20 @ 35% CE, 288.20 @ 35% CE: 147.0 (12.4), 142.12(9.3), 124.0 (100).

3.7.3. E-Thesinine-O-4′-α-rhamnoside-glycoside

ESIMS (positive ion mode; m/z, rel int (%)) 596.3 $[MH]^+$ ms^2 596.30 @ 35% CE: 434.2 (3.4), 288.2 (100), ms^3 596.3 @ 35% CE, 288.20 @ 35% CE: 147.0 (14.4), 142.12 (19.3), 124.0 (100).

3.7.4. Z-Thesinine-O-4′-α-rhamnoside-glycoside

ESIMS (positive ion mode; m/z, rel int (%)) 596.3 $[MH]^+$ ms^2 596.30 @ 35% CE: 434.2 (3.4), 288.2 (100), ms^3 596.3 @ 35% CE, 288.20@ 35% CE: 147.0 (12.4), 142.12 (8.3), 124.0 (100).

3.7.5. E- and Z-p-coumaric acid-4-O-α-rhamnoside

Analytical HPLC: Rt = 16.7 min and 18.5 min; ESIMS (negative ion mode; m/z, rel int (%)) 309.1 $[M–H]^-$, ms^2 309.1 @ 35% CE: 163.0 (100), 119.1 (6), ms^3 @ 35% CE: 119.1 (100) for both peaks.

3.7.6. (+)-Isoretronecanol

$[\alpha]_D^{26}$ +55° (EtOH; *c* 0.055) Lit. $[\alpha]_D$ +72° (Chapman and Hall Dictionary of Natural Products, 2004); GC-MS: R_I (DB-1MS), 1266; m/z 141 $[M^{\cdot}]^+$.

3.7.7. Trachelanthamidine

GC-MS: R_I (DB-1MS), 1239; m/z 141 $[M^{\cdot}]^+$.

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