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GLYCOSIDASE-INHIBITING PYRROLIDINE ALKALOIDS FROM *HYACINTHOIDES NON-SCRIPTA*

ALISON A. WATSON, ROBERT J. NASH,* MARK R. WORMALD,† DAVID J. HARVEY,† STEPHEN DEALLER,‡ EILEEN LEES,‡ NAOKI ASANO,§ HARUHISA KIZU,§ ATSUSHI KATO,§ RHODRI C. GRIFFITHS, ANDREW J. CAIRNS and GEORGE W. J. FLEET¶

Chemistry Group, Institute of Grassland and Environmental Research, Plas Gogerddan, Aberystwyth, Cardiganshire SY23 3EB, U.K.; † Glycobiology Institute, Biochemistry Department, South Parks Road, Oxford OX1 3QU, U.K.; ‡ Microbiology Department, Burnley General Hospital, Casterton Avenue, Burnley BB10 2PQ, U.K.; § Faculty of Pharmaceutical Sciences, Hokuriku University, Kanazawa 920-11, Japan; ¶ Dyson Perrins Laboratory, South Parks Road, Oxford OX1 3QY, U.K.

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Key Word Index—*Hyacinthoides non-scripta*; Hyacinthaceae; bluebell; alkaloids; (2*R*,3*R*,4*R*,5*R*)-2,5-dihydroxymethyl-3,4-dihydroxypyrrolidine; 2,5-dideoxy-2,5-imino-DL-*glycero*-D-*manno*-heptitol; 2,5-dideoxy-2,5-imino-DL-*glycero*-D-*manno*-heptitol-7-*O*-apioside; 1,4-dideoxy-1,4-imino-D-arabinitol; glycosidase inhibition; livestock poisoning.

Abstract—The glycosidase-inhibiting pyrrolidine alkaloids (2*R*,3*R*,4*R*,5*R*)-2,5-dihydroxymethyl-3,4-dihydroxypyrrolidine (DMDP), 2,5-dideoxy-2,5-imino-DL-*glycero*-D-*manno*-heptitol (homoDMDP), homoDMDP-7-*O*-apioside and 1,4-dideoxy-1,4-imino-D-arabinitol have been identified in the leaves of bluebells (*Hyacinthoides non-scripta*). HomoDMDP and homoDMDP-7-*O*-apioside are new natural products. Glycosidase inhibition by the aglycones is compared and could explain the symptoms of poisoning of livestock by bluebells. © 1997 Elsevier Science Ltd

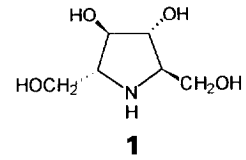
INTRODUCTION

Although rare, there are reports of livestock which have been poisoned by grazing on bluebells, *Hyacinthoides non-scripta*. The symptoms in the horse are abdominal pain and dysentery, whereas cows suffer from lethargy and dullness [1]. The toxic principles remained undescribed but we have now identified nitrogen analogues of mono- and disaccharides, which are related to glycosidase-inhibiting alkaloids, such as swainsonine, that have been implicated in livestock poisonings [2, 3]. This is the first report of such alkaloids in the Hyacinthaceae.

RESULTS AND DISCUSSION

The polar fraction of an 80% ethanolic extract of *H. non-scripta* leaves subjected to cation-exchange chromatography (Dowex 50X8 H^+ form) at 10 mg (equivalent to fr. wt of original material) ml^{-1} gave 100% inhibition of α-glucosidase activity in a homogenate of cow brain and 70% inhibition of the β-glucosidase and β-galactosidase activities. At the same concentration, the sample inhibited the β-*N*-acetylgalactosaminidase and β-*N*-acetylglucosaminidase activities by 33 and 26%, respectively. GC-mass spectrometric analysis of the trimethylsilylated extract revealed a number of alkaloids to be present, the major one being (2*R*,3*R*,4*R*,5*R*)-2,5-dihydroxymethyl-3,4-dihydroxypyrrolidine (DMDP, **1**) which has been reported to be a potent inhibitor of α- and β-glucosidases from several sources [4, 5]. Another glycosidase-inhibiting alkaloid, 1,4-dideoxy-1,4-imino-D-arabinitol **2** [6], was also detected in the extract but, in addition, there were several novel alkaloids and glycosides of these alkaloids. This is the first report of **1** and **2** from British plants. One of the major alkaloids detected by GC-mass spectrometry (TMSi-) showed a striking resemblance to the synthetic compound α-homoDIM (2,5-dideoxy-2,5-imino-D-*glycero*-D-talo-heptitol) (**3**) [7] (with distinctive ions at *m/z* 538 5% $[M-Me]^+$, 450 30% $[M-Me+OSiMe_3]^+$, 348 100% $[M-Me+OSiMe_3+CH_2OSiMe_3]^+$ and 217 80%), but with a shorter retention time (8.8 *vs* 10.2 min). It was determined to be 2,5-dideoxy-2,5-imino-DL-*glycero*-D-*manno*-heptitol (homoDMDP, **4**) but the absolute configuration is unknown. One of the glycosides was also purified and found on acid hydrolysis to be the apioside **5** of homoDMDP.

* Author to whom correspondence should be addressed.



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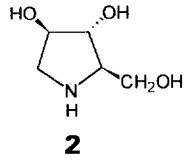

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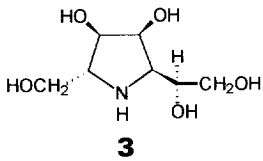

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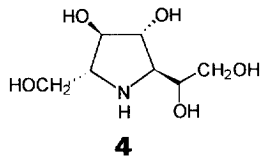

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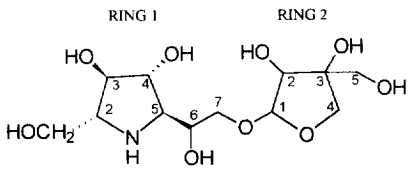

5

The ^{1}H NMR spectrum of **4** in ^2H_2O contains nine non-equivalent, non-exchanging protons and seven carbons. The proton chemical shifts (δ), multiplicities and three-bond coupling constants ($^3J_{HH}$), determined from the 1D and 2D ^{1}H–^{1}H COSY spectra, and carbon chemical shifts and multiplicities, determined from the 1D and 2D ^{1}H–^{13}C HMQC spectra, for the sample in ^2H_2O are given in Table 1. The 2D COSY spectrum shows a single linear sequence of $^3J_{HH}$ correlations from C–1H/H′ to C-7H/H′ in the order given in the Table 1, defining a linear CH_2–$(CH)_5$–CH_2 carbon backbone. This accounts for all the carbons and the non-exchanging protons. From inspection of the ^{1}H and ^{13}C chemical shift data, C-1, C-3, C-4, C-6, and C-7 must also be bonded to oxygen, and C-2 and C-5 to nitrogen. Because of the chemical shift dispersion and range of coupling constants observed, the compound must be cyclic, most probably *via* the nitrogen to give a five-membered ring. The 1D spectrum in DMSO shows several exchangeable protons. There are two downfield hydroxyl resonances at δ 4.83 and δ 4.73, which can be assigned to the C-3 and C-4 carbons, respectively, from the COSY spectrum. At 30°, there is severe overlap between the other exchangeable proton resonances and those from C-1H/H′, C-6H and C-7H/H′ (δ 3.2–3.5). At 65°, some of these resonances shift to a clear region of the spectrum, but chemical exchange becomes too fast to allow assignments to be made. In the glycoside described below, hydroxyl protons can also be assigned to C-1 and C-6, confirming the hetero-atom bridge between C-2 and C-5. The resulting covalent structure is identical to that of the synthetic compound α-homoDIM (**3**). The pattern of ^{1}H and particularly ^{13}C chemical shifts for **4** is very similar to that of **3** (comparison given in Table 3), consistent with the aglycone being

Table 1. ^{1}H and ^{13}C assignments and coupling constants for homoDMDP (**4**) in ^2H_2O, pH = 7.60 and 30°. Numbering scheme is shown in **5** (ring 1)

Label	^{1}H δ	Mult.	$^3J_{HH}$ (Hz)	^{13}C δ	Mult.
C-1	3.74	*dd*	11.8/3.8	60.6	CH_2
	3.69	*dd*	11.9/6.1		
C-2	3.16	*broad*	—	61.7	CH
C-3	3.91	*t*	7.3	77.0	CH
C-4	4.12	*t*	7.3	76.7	CH
C-5	3.13	*broad*	—	61.3	CH
C-6	3.84	*broad*	—	71.7	CH
C-7	3.74	*dd*	11.8/3.8	62.9	CH_2
	3.64	*dd*	11.7/6.9		

Table 2a. ^{1}H and ^{13}C assignments and coupling constants for the apioside **5** in ^2H_2O, pH = 7.70 and 30°

Label	^{1}H δ	Mult.	$^3J_{HH}$ (Hz)	^{13}C δ	Mult.
1C-1	3.73	*dd*	11.9/4.3	60.6	CH_2
	3.68	*dd*	11.9/5.8		
1C-2	3.08	*broad*	—	61.2	CH
1C-3	3.88	*t*	7.5	76.9	CH
1C-4	4.12	*t*	7.5	76.9	CH
1C-5	3.07	*broad*	—	60.7	CH
1C-6	3.92	*broad*	—	70.4	CH
1C-7	3.88	*dd*	10.7/3.3	69.7	CH_2
	3.64	*dd*	10.7/7.2		
2C-1	5.10	*d*	3.4	108.2	CH
2C-2	4.02	*d*	3.4	75.8	CH
2C-3	*n/a*	*n/a*	n/a	78.5	C
2C-4	4.08	*d*	10.3	72.8	CH_2
	3.90	*d*	10.3		
2C-5	3.68	*s*	n/a	62.7	CH_2

Table 2b. ^{1}H assignments and coupling constants for the apioside **5** in DMSO, 30°

Label	C^{1}H δ	Mult.	$^3J_{HH}$ (Hz)	O^{1}H δ
1C-1	3.42	*broad*	—	4.50
	3.29	*broad*		
1C-2	2.75	*m*	—	*n/a*
1C-3	3.52	*m*	—	4.82
1C-4	3.77	*m*	—	4.70
1C-5	2.76	*m*	—	*n/a*
1C-6	3.57	*broad*	—	4.61
1C-7	3.64	*dd*	10.5/3.0	*n/a*
	3.30	*dd*	10.5/7.8	
2C-1	4.80	*d*	3.2	*n/a*
2C-2	3.74	*broad*	—	5.12
2C-3	*n/a*	*n/a*	*n/a*	{4.52}
2C-4	3.85	*d*	9.3	*n/a*
	3.56	*d*	9.3	
2C-5	3.32	*m*	—	4.80

{...}—tentative assignment.

Table 3. Comparison of ^1H and ^{13}C assignments for homoDMDP **4**, the apioside **5** (ring 1) and α-homoDIM **3** in ^2H_2O, 30°

	^1H			^{13}C		
Label	**4**	**5** ring 1	**3**	**4**	**5** ring 1	**3**
C-1	3.74	3.73	3.85	60.6	60.6	60.2
	3.69	3.68	3.74			
C-2	3.16	3.08	3.33	61.7	61.2	61.4
C-3	3.91	3.88	4.12	77.0	76.9	72.3
C-4	4.12	4.12	4.31	76.7	76.9	71.2
C-5	3.13	3.07	3.35	61.3	60.7	59.7
C-6	3.84	3.92	3.95	71.7	70.4	69.0
C-7	3.74	3.88	3.77	62.9	69.7	62.9
	3.64	3.64	3.62			

a diastereoisomer of **3**. Both the C-3H and C-4H resonances are triplets with 7.3 Hz coupling constants (compared with a 3.8/9.0 Hz doublet of doublets and a 3.5 Hz triplet, respectively, for **3**), indicating that the protons H-2, H-3, H-4 and H-5 are all *trans*. This gives the structure as **4** or its enantiomer. The relative configuration at C-6 cannot be determined from the NMR data ($[\alpha]_D^{25}$: +31.5° *c* 0.44, water).

Compound **5**, approximately 85% pure, containing 13 non-equivalent and two equivalent, non-exchanging protons and 12 carbons. The protons chemical shifts (δ), multiplicities and three-bond coupling constants for the sample in ^2H_2O are given in Table 2(a). The M_r of the glycoside, as measured by MALDI mass spectrometry, is 325.4, consistent with an empirical formula of $C_{12}H_{23}O_9N$. The 2D COSY spectrum shows a linear sequence of correlations from 1C-1H/H′ to 1C-7H/H′ (labelled ring 1) in the order given in Table 2(a). This sequence is confirmed by the $^2J_{CH}$ and $^3J_{CH}$ couplings observed in the 2D ^1H–^{13}C HMBC spectrum. The pattern of ^1H and ^{13}C chemical shifts (comparison given in Table 3) and $^3J_{HH}$ coupling constants is almost identical to **4** (except for the C-7 ^{13}C resonance). The 2D COSY spectrum in DMSO [Table 2(b)] allows assignment of hydroxyl protons to the 1C-3 and 1C-4 carbons, whilst the 2D TOCSY spectrum allows the further assignment of hydroxyl protons to the 1C-1 and 1C-6 carbons. The only other correlations observed in the 2D COSY spectrum are between 2C-1H...2C-2H and 2C-4H...2C-4H′. Thus, the carbon backbone structure of ring 2 has to be determined from the pattern of $^3J_{CH}$ correlations observed in the HMBC spectrum. Correlations are observed between 2C-1...2C-4H/H′ and 2C-4...2C-1H and between 2C-2...2C-4H′. This indicates the presence of a five-membered ring, the 2C-1 to 2C-4 and 2C-2 to 2C-4 linkages involving atoms with no non-exchangeable protons (either oxygen or the quaternary carbon, the only nitrogen already having been accounted for in the aglycone). The 2C-1 ^1H and ^{13}C chemical shifts are typical of a monosaccharide anomeric carbon, thus the 2C-1 to 2C-4 linkage must be *via* a ring oxygen and the 2C-2 to 2C-4 linkage must be *via* the quaternary carbon (2C-3). This is supported by the correlations observed between 2C-3...2C-4H/H′. Correlations are also seen between 2C-4...2C-5H, 2C-5...2C-3H and 2C-5...2C-4H; thus, 2C-5 must be attached to 2C-3. This is confirmed by NOEs between 2C-2H...2C-5H and 2C-4H...2C-5H. The 2D COSY spectrum in DMSO allows assignment of a hydroxy proton to the 2C-2 carbon, whilst the 2D TOCSY spectrum allows the assignment of a hydroxyl proton to the 2C-5 carbon and the tentative assignment to the 2C-3 carbon (*via* the $^4J_{HH}$ couplings to 2C-2H and 2C-4H/H′), confirming the ring structure suggested above (**5**, ring 2). It is interesting to note that very strong TOCSY (or exchange) peaks are observed between 2C-2OH...2C-5H and 2C-2OH...2C-5OH suggesting possible hydrogen-bonding involving these two hydroxyl groups. The linkage between ring 1 and ring 2 of **5** is defined by the HMBC correlations between 2C-1...1C-7H/H′ and 1C-7...2C-1H, giving a glycosidic linkage between 1C-7 and 2C-1. This is confirmed by NOEs between 2C-1H...1C-7H/H′. The homoDMDP-7-*O*-apioside **5** is the first glycoside of this type to be reported. The branched-chain sugar apiose is of interest, in that little is known about its biological functions [8]. The glycoside was not pure enough for glycosidase assays.

Compounds **1**–**3** are potent inhibitors of glycosidases [4–7]. Compound **4** was tested on a range of glycosidases and is compared using IC_{50} values with **1** and **2** in Table 4. **4** is a more potent inhibitor (K_i = 1.5 μM) of almond β-glucosidase than **1** (K_i = 10 μM) and **2** (K_i = 280 μM). However, only **2** inhibited rabbit gut β-glucosidase activity at mM concentrations and, similarity, it was the only compound to inhibit rabbit gut α-glucosidase activity; all of the compounds significantly inhibited yeast α-glucosidase. In preliminary disaccharidase assays using rabbit gut activities (at concentrations of 875 μM **1**, 1 mM **2** and 740 μM **4**), all three alkaloids inhibited sucrase but only **4** appeared to inhibit lactase and maltase; **1** and **4** also inhibited trehalase. Only **1** inhibited xylanase from *Trichoderma viride*. **1** has been reported to be a weak inhibitor of yeast invertase [4] and, in the present

Table 4. Comparison of glycosidase inhibition of pyrrolidine alkaloids from *Hyacinthoides non-scripta*

	IC_{50} (μM)		
Glycosidase	**1**	**2**	**4**
α-Glucosidase (baker's yeast)	15	7.0	85
α-Glucosidase (rabbit gut)	NI	85	NI
β-Glucosidase (almond)	9	965	4
β-Glucosidase (rabbit gut)	NI	LI	NI
α-Mannosidase (jack bean)	NI	750	695

NI, no inhibition at 875 μM (**1**), 1 mM (**2**) and 740 μM (**4**)
LI, inhibition below 50% at 1 mM.

study, **1** and **4** were found to be inhibitory to *Phleum pratense* invertase activity with K_i values of 78 and 77 μM, respectively. **2** was a weaker inhibitor with a K_i of 1.07 mM.

The alkaloids were not inhibitory to α-galactosidase (green coffee bean), β-glucosidase (rabbit liver), β-*N*-acetylglucosaminidase (bovine and jack bean) or naringinase (*Pencillium decumbens*). **1** and **4** were weakly inhibitory (below 40%) to β-galactosidase activities (rabbit gut and liver) at the concentrations described above. None of the alkaloids inhibited cellulase or pectinase (both from *Aspergillus niger*) *at* 300 μM (**1**), 260 μM (**2**) and 375 μM (**4**).

Inhibition of glycosidase activities by the polyhydroxylated pyrrolidine alkaloids purified from bluebells suggests that these compounds could be responsible for the toxicity of this species to mammals. In addition to the alkaloids identified here, there are also other related alkaloids detected in the leaves by GC-mass spectrometry and these may contribute to the inhibition of bovine brain enzymes shown by the partially purified extract of bluebell leaves. The mannosidase-inhibiting alkaloid, swainsonine, found in *Swainsona*, *Astragalus*, *Oxytropis* and *Ipomoea* species [9], causes depression, tremors, emaciation and gastrointestinal malfunction in cattle, with a conservative estimate of the toxic dose in the diet being as low as 0.001% [3]. However, glycosidase-inhibiting alkaloids have also aroused considerable interest in recent years as potential chemotherapeutic agents for use against cancers, viruses and diabetes [10]. For example, swainsonine, when given orally at low concentrations, has a significant antimetastatic effect, which appears to be largely due to augmentation of the immune system [11]. Many of the potential medical applications of these alkaloids seem to be the result of inhibition of specific glycosidases involved in the formation of glycoprotein oligosaccharide chains and the discovery of new biologically-active alkaloids and glycosides in the bluebell may result in a greater range of potential applications.

The identification of **1** in the bluebell is of interest because it has previously only been reported from tropical plant species and a *Streptomyces* species [10]. This alkaloid has been shown to be inhibitory to several plant parasitic nematode species [12] and **1** and **2** have also been reported to be antifeedants to insects [10]. The presence of a number of glycosidase inhibitors in bluebell leaves and bulbs (in which they occur at similar concentrations) may therefore act as a defence to a number of classes of potential predators.

EXPERIMENTAL

Plant material. Bulbs of *Hyacinthoides non-scripta* (L.) Chouard ex Rothm. were donated by O. A. Taylor and Sons Bulbs Ltd of Spalding, U.K. The bulbs were grown at the Institute of Grassland and Environmental Research (IGER) and a voucher specimen (Nash96002) is held at the Herbarium at IGER.

Extraction and isolation. Fr. *H. non-scripta* leaves (800 g) were homogenised in 80% aq. EtOH (200 mg ml^{-1}) and left to extract for 24 hr at 4°. After filtering, alkaloids and basic amino acids were isolated on cation-exchange resin (Dowex 50-X2 14–50 mesh H^+ form) in a column (20 × 3 cm), displacing with 2 M NH_4OH after washing with 2 l of 2 M pyridine. GC-MS of the TMSi-alkaloid fr. showed the major alkaloid to be **1** but several related alkaloids and glycosides were also present. Free bases were sepd using the cation-exchange resin Amberlite CG120 (200–400 mesh) in the NH_4^+ form by elution with 0.1 M NH_4^+ with the glycosides eluted first, then **1** (67 mg), **4** (7.2 mg) and, finally, **2** (9 mg). The structures of **1** and **2** were confirmed by ^1H NMR by comparison with authentic samples. One of the glycosides **5** was purified by chromatography on neutral aluminium oxide (Merch 90 active I) in Me_2CO with increasing H_2O concns (glycoside obtained in 40% Me_2CO, yield 5.2 mg). Acid hydrolysis of **5** and GC-MS analysis revealed the aglycone to be **4**.

GC-MS. TMSi-derivatives of the alkaloids and glycosides were produced for GC-MS using Sigma Sil A (50 μl mg^{-1}). The GC column was a 25 m × 0.25 mm BPX5 (SGE) with a temp. programme of 180–300° with a rise of 10° min^{-1}. The MS was operated at 70 eV with mass detection of 100–650 mu. The R_ts (min) were 3.9 (**2**), 6.2 (**1**), 8.8 (**4**), 10.2 (**3**) and 18.3 (**5**).

MS. Matrix-assisted laser desorption/ionisation (MALDI) mass spectrometry was performed as described earlier [13]. The ion source was a standard FAB source with the N_2 laser beam entering through a sapphire window in the top of the ion-source chamber. Laser targets were prepd by mixing aq. sample of **5** (*ca* 100 pmole) with 2,5-dihydroxybenzoic acid (500 nmole from a saturated soln in MeCN) on the mass spectrometer side-entry probe and allowing the mixt. to crystallise. The residue was then recrystallised from EtOH (*ca* 0.2 μl). During spectral acquisition the laser was run at full power (180 μJ $pulse^{-1}$) in order to achieve maximum sensitivity. The laser was fired at a repetition rate of 20 shots sec^{-1} and one-second exposures were acquired with the array detector. The mass range of m/z 200–1000 was achieved by stepping the magnet, under data-system control to give the required number of exposures (array ratio 1.2:1). To compensate for the depletion of sample under the laser spot, the laser beam was scanned manually across the target by means of a micrometer attached to the mirror that directed the laser beam at the ion source. Several complete spectra were acquired and averaged in order to accommodate fluctuations in ion intensity from different areas of the target.

NMR. All spectra were recorded on a Varian Unity 500 spectrometer. Samples dissolved in ^2H_2O were all adjusted to pH 7.6 to 7.7 and all spectra recorded at a probe temp. of 30°. Spectra of samples in DMSO were recorded at both 30 and 65°. All 2D-spectra (^1H–^1H COSY, TOCSY and NOESY, ^1H–^{13}C HMQC and

HMBC) were acquired in phase-sensitive mode. TOCSY spectra were recorded with an 8 kHz spin-lock field and a 40 ms mixing-time. NOESY spectra were recorded with mixing times of 100 to 500 ms without any random variation in mixing time. For samples in ^2H_2O, all ^1H chemical shifts are referenced to Me_2CO at δ 2.235, ^{13}C chemical shifts to Me_2CO at δ 29.8. For samples in DMSO, ^1H chemical shifts are referenced to the residual solvent signal at δ 2.49.

Glycosidase assays. Activity against a range of commercially available glycosidases (Sigma) was assayed at a microtitre scale at the pH optimum for each enzyme. The incubation mixt. was 20 μl enzyme soln (at appropriate concn), 20 μl inhibitor soln and 100 μl *p*-nitrophenylglycopyranosides (5 mM). Enzyme and inhibitor solns were preincubated for 15 min at 30° before starting the reaction by addition of substrate. The reaction was stopped after 10 min by addition of 160 μl glycine buffer (1M, pH 10.4) and the *A* read at 405 nm. Assays of rabbit gut and liver glycosidase activities were conducted using white lab rabbits killed by cervical dislocation. The small intestines and liver were removed and flushed with ice-cold isotonic saline. The small intestines were opened lengthwise and the mucosa scrapped off, diluted with distilled H_2O and centrifuged at 4° for 10 min at 3020 *g*. Proteins were pptd using $(NH_4)_2SO_4$, the pellets being dissolved in NaH_2PO_4 (50 mM, pH 6.5) and used for the assays. The liver was homogenised and then treated as described for gut. Activity was tested using the same incubation mixt. as described above, replacing commercial enzyme with homogenate. Rabbit gut disaccharide assays (sucrase, lactase, maltase and trehalase) used the same gut prepn and an assay method based on that of ref. [14]. The incubation mixt. of 10 μl homogenate, 10 μl inhibitor soln and 20 μl of substrate (56 mM in NaH_2PO_4, pH 6.5) was incubated at 30° for 12 hr. Cellulase, pectinase and xylanase were assayed by a modified neocuprione assay for reducing sugar [15]. Invertase activity in Timothy grass (*Phleum pratense* L., cv. S352) [16] was determined as reducing sugar released from sucrose [17] in the concn range 10–90 mM at 2.0 nkat ml^{-1}, pH 5.5 (in McIlvaine buffer, 20 mM) and 30°, in the presence and absence of 300 and 600 μM of inhibitor; K_i values were obtained by Lineweaver–Burk analysis [18]. A crude prepn of *H. non-scripta* leaves after purification using Dowex 50 (H^+ form) was also included in preliminary glycosidase inhibition assays using crude homogenates of bovine brain material collected from abattoirs. The assays used *p*-nitrophenyl (α-glucosidase)- and methylumbelliferyl substrates. The activities screened were α-glucosidase, β-glucosidase, β-galactosidase, β-*N*-acetylglucosaminidase and β-*N*-acetylgalactosaminidase. The crude plant prepn strongly inhibited all enzyme activities when equivalent to 100 mg (fr. wt of original material) ml^{-1} but the selectivity of the inhibition profile was evident when this crude prepn was diluted at least 1:10.

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