Structure Elucidation of Monatin, a High-intensity Sweetener Isolated from the Plant *Schlerochiton ilicifolius*

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The structure of monatin, a high-intensity sweetener isolated from *Schlerochiton ilicifolius* was elucidated by ^1H and ^{13}C NMR spectroscopy as 4-hydroxy-4-(indol-3-ylmethyl)glutamic acid. The 2*R**,4*R** relative configuration of the two chiral centres in monatin was determined by NOE studies on a derivative, methyl 2-(indol-3-ylmethyl)-4-(2,4-dinitroanilino)-5-oxo-2,3,4,5-tetrahydrofuran-2-carboxylate. The assignment of the 2*S* configuration to monatin, through application of the Clough–Lutz–Jirgenson rule, established the 2*S*,4*S* configuration for the compound.

The detrimental effects of high consumption of sucrose and glucose-based sugars, such as obesity and tooth decay in humans,[1,2] have resulted in the present day extensive research on synthetic, non-nutritive sweeteners with the aim to develop a safe, high-intensity, non-calorific sweetener, stable for a wide range of pH and temperatures, with a long shelflife and no aftertaste. None of the present products on the market, *e.g.* saccharin, cyclamate, acesulfame-K and aspartame fulfil all these requirements.[3] The concern about long-term effects of some of these products has resulted in a search for high-intensity sweeteners from natural sources.[4] Three of the more promising products are thaumatin, a 22 209 dalton protein obtained from African katemfe fruit, *Thaumatococcus danielli*,[5] glycyrrhizin, a glycoside extracted from the roots of *Glycyrrhiza glabra*,[6] and stevioside, another glycoside obtained from the herb, *Stevia rebaudiana*.[7]

The rocky hills in the north-western Transvaal are the habitat of a spiny-leaved hardwood shrub, *Schlerochiton ilicifolius*, that grows to a height of about 2 m. The high-intensity sweetener isolated from the bark of the roots in our study on *S. ilicifolius* was named monatin from the Sepedi name 'monate' meaning nice.

The air-dried, ground roots of *S. ilicifolius* were extracted with water and the extracts treated with AG50W-X8 cation ion exchange resin (H^+ form). The basic compounds bound to the resin were collected by stirring with aqueous ammonia. The combined ammonia extracts were freeze-dried and the residual material purified by gel filtration on consecutively (i) Biogel P2 and (ii) Sephadex G10 (2×). The sweet tasting fractions were combined and freeze-dried to yield monatin, $[\alpha]_D^{20}$ −49.6, as a mixture of salts in which the sodium salt predominated (>95%). Treatment of an aqueous solution of monatin salt with acetic acid followed by the addition of ethanol gave the free acid of monatin **1** as fine needle-like crystals, m.p. 216–220 °C, which analysed for $C_{14}H_{16}N_2O_5 \cdot \frac{1}{4}H_2O$. The compound gave positive Ehrlich and ninhydrin colour reactions indicative of the presence of a 2-unsubstituted indole and an amino acid moiety, respectively. The UV spectrum of monatin was typical of an indole and had λ_{max} 279 (ε 5455).

The structure elucidation of monatin is based on a detailed study of the highfield ^1H and ^{13}C NMR spectral data of the compound (Table 1). The signal at δ_H 7.192 (s) was assigned to the C-2 proton of the putative indole moiety. The proton–proton connectivity pattern followed from the first-order analysis of the remaining multiplets as well as $^1H\{^1H\}$ decoupling experiments and established the presence of three fragments A, B and C in monatin.

Fragment A. The proton chemical shifts and coupling constants of this four-proton spin system are typical of the protons of an indole moiety.[8]

Fragment B. The chemical shift values of the resonances at δ_H 3.243 (10-H_a) and 3.051 (10-H_b) and the coupling constant (*J* 14.3 Hz) of this isolated two-proton spin system suggest that C-10 is bonded to an sp^2 hybridised carbon atom.

Fragment C. The signal at δ_H 3.168 (1 H, s, *J* 11.6 and 1.8) serves as the terminus of this three-proton ABX spin system and is indicative of the methine proton of an amino acid. The chemical shifts of the AB part of the spin system (δ_H 2.651 and 2.006) are in agreement with the values reported for the β-methylene protons of amino acids.[9]

The multiplicities of the 14 different ^{13}C resonances were deduced from the coupled ^{13}C NMR spectrum. The ^{13}C resonances were partly assigned by correlation of the proton-bearing carbon atoms with specific proton resonances in a two-dimensional (^{13}C, ^1H) chemical shift correlation experiment.[10,11] The connections between the different structural units followed from the two- and three-bond (^{13}C, ^1H) connectivity pattern established in a number of heteronuclear $^{13}C\{^1H\}$ selective population inversion (SPI) experiments (see Table 2).[12]

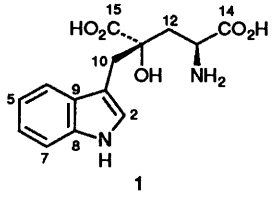
The resonance at δ_C 126.03 (d) which correlates with the signal at δ_H 7.192 (s) is assigned to the C-2 proton of the indole ring on the basis of the chemical shift value and the one-bond (^{13}C, ^1H) coupling constant [^1J(CH) 182.2 Hz]. Application of a π-pulse at a position 5.0 Hz to lowfield of the 2-H resonance in an SPI experiment affected the resonances at δ_C 110.31 (s, C-3), 137.06 (s, C-8) and 129.23 (s, C-9). The assignment of these resonances is based on a comparison with the chemical shift values of other indole compounds, *e.g.* tryptophan.[13] The link between fragment B and the indole ring was established by the (^{13}C, ^1H) connectivity pattern determined for the C-10 methylene protons in an SPI experiment (see Table 2). Irradiation in separate experiments of each of the C-10 protons affected the resonances assigned to C-2, C-3 and C-9 and locates the C-10 methylene group at C-3 of the indole ring. In addition the C-10 methylene protons must be linked to quaternary carbon atoms two- and three-bonds removed as the resonances at δ_C 81.41 (s) and 181.18 (s) are also affected in these SPI experiments. The link between fragments B and C was established by the correlation observed between the C-12 methylene protons and the resonance at δ_C 81.41 (s, C-11) in an SPI experiment. On the basis of the two- and three-bond (^{13}C, ^1H) connectivity pattern the structure as shown in **1** is assigned to monatin.

A less likely alternative structure **2** for monatin in which the hydroxy and amino groups are interchanged, is excluded for a number of reasons. Although the chemical shift values of the

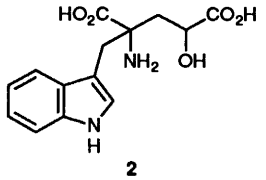
Table 1 ^1H and ^{13}C NMR data for monatin salt **1** and the derivative **3**

	1				**3**		
Atom	δ_C[a]	J(CH)/Hz	δ_H[b]	J(HH)/Hz	δ_C[c]	δ_H[c]	J(HH)/Hz
2	126.03 (d)	182.2	7.192 (s)		126.42 (d)	7.371 (s)	—
3	110.31 (s)	—	—	—	108.12 (s)	—	—
4	120.46 (d)	159.0	7.686 (d)	7.9	120.43 (d)	7.804 (d)	7.9
5	120.25 (d)[d]	159.4	7.102 (dd)[e]	8.0, 8.0	119.49 (d)	7.180 (dd)	7.9, 8.0
6	122.74 (d)[d]	159.6	7.176 (dd)[e]	8.0, 8.0	122.84 (d)	7.235 (dd)	8.0, 8.1
7	112.79 (d)	160.3	7.439 (d)	8.1	112.72 (d)	7.502 (d)	8.1
8	137.06 (s)	—	—	—	137.59 (s)	—	—
9	129.23 (s)	—	—	—	132.08 (s)	—	—
10	36.53 (t)	128.2	3.243 (d)	14.3	33.58 (t)	3.513 (d)	14.9
			3.051 (d)	14.3		3.599 (d)	14.9
11	81.41 (s)	—	—	—	86.01 (s)	—	—
12	39.31 (t)	130.2	2.651 (dd)	15.3, 1.7	36.79 (t)	2.775 (dd)	10.3, 13.2
			2.006 (dd)	15.3, 11.7		3.195 (dd)	9.2, 13.1
13	54.89 (d)	144.2	3.168 (dd)	11.6, 1.8	53.22 (d)	3.916 (m)	
14	175.30 (s)	—	—		171.47 (s)[f]	—	—
15	181.18 (s)	—	—		172.94 (s)[f]	—	—
16					—	6.371 (d)	9.5
17					126.26 (s)[g]	—	—
18					128.95 (s)[g]	—	—
19					124.17 (d)	8.918 (d)	2.6
20					128.28 (s)[g]	—	—
21					130.64 (d)	8.088 (dd)	2.5, 9.4
22					115.27 (d)	8.743 (d)	9.4
OMe					53.21 (q)	3.798 (s)	—

[a] Relative to internal dioxane at δ_C 67.80; solvent D_2O. [b] Relative to internal dioxane at δ_H 3.700; solvent D_2O. [c] Solvent [$^2H_6$]acetone. [d–g] May be interchanged.



1



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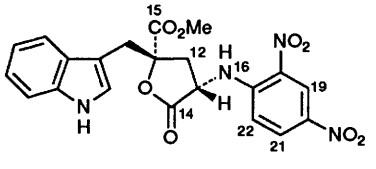

3

C-11 and C-13 resonances [δ_C 81.41 (s) and 54.89 (d)] militate against structure **2** the value of J(CH) of 144.2 Hz for C-13 favours either structure.

Additional evidence in favour of structure **1** was obtained from the pH dependence of the ^1H chemical shifts of monatin. The ^1H chemical shift of the α-proton of α-amino acids is pH dependent.[14] Under basic conditions this proton resonance shifts upfield and under acidic conditions it moves downfield compared to its position under neutral conditions. Although the other protons behave in a similar fashion the effect is less pronounced. The data for selected protons of monatin are collated in Table 3. From Table 3 it can be seen that 13-H of structure **1** appears at δ_H 3.618 (dd, J 11.6 and 1.8 Hz) but moves upfield by 0.781 ppm at pH 13 and downfield by 0.349 ppm at pH 1. These pH dependent shifts can not be accommodated by the alternative structure **2** and indicated the structure **1** for monatin. Although the C-12 protons also experience substantial shifts at pH 13 this is due to the cumulative effect of the two carboxylic acid groups.

A derivative of monatin was prepared by reaction of the amino group of monatin with Sanger's reagent (2,4-dinitrofluorobenzene) to give the *N*-2,4-dinitrophenyl (DNP) derivative. The product was converted into a dimethyl ester by treatment with an ether solution of diazomethane. Elemental analysis and accurate mass determination of the molecular ion at m/z 454, gave the molecular formula of the ester **3** as $C_{21}H_{18}N_4O_8$. This molecular formula as well as the resonances at δ_H 3.798 (s) and δ_C 53.21 (q) in the ^1H and ^{13}C NMR spectra (see Table 1) indicates the presence of a single methyl ester

Table 2 Two- and three-bond (^{13}C, ^1H) connectivity pattern for monatin salt **1**

δ_H			Correlation signals δ_C	
1	7.192	(2-H)	110.31 (s)	(C-3)
			129.23 (s)	(C-9)
			137.06 (s)	(C-8)
2	3.243	(10-H_a)	129.23 (s)	(C-9)
			126.03 (d)	(C-2)
			110.31 (s)	(C-3)
			81.41 (s)	(C-11)
3	3.051	(10-H_b)	181.18 (s)	(C-15)
			129.23 (s)	(C-9)
			126.03 (d)	(C-2)
			110.31 (s)	(C-3)
			81.41 (s)	(C-11)
4	2.651	(12-H_a)	175.30 (s)	(C-14)
			81.41 (s)	(C-11)
			54.89 (d)	(C-13)
5	2.006	(12-H_b)	181.18 (s)	(C-15)
			81.41 (s)	(C-11)
			54.89 (d)	(C-13)
6	3.168	(13-H)	175.30 (s)	(C-14)
			81.41 (s)	(C-11)
			39.31 (t)	(C-12)

Table 3 pH Dependence of the chemical shifts of monatin **1**

	δ_H				
pH	10-H_a	10-H_b	12-H_a	12-H_b	13-H
Neutral	3.243	3.051	2.651	2.006	3.618
pH1	3.245	3.084	2.618	2.083	3.967
$\Delta\delta^a$	−0.002	−0.033	0.033	−0.077	−0.349
pH 13	3.077	2.907	2.237	1.597	2.837
$\Delta\delta^b$	0.166	0.144	0.414	0.409	0.781

[a] $\Delta\delta$ = δ(neutral) − δ(pH 1). [b] $\Delta\delta$ = δ(neutral) − δ(pH 13).

group in the product **3**. The formation of the product **3** can be rationalised by the involvement of the hydroxy group in the formation of a lactone ring by reaction with either the C-11 or the C-13 methoxycarbonyl group, depending on the structure **1** or **2** proposed for monatin. The appearance of the 16-NH proton as a doublet (J 9.5 Hz) at δ_H 6.371 as a result of vicinal coupling with the C-13 proton confirms the structure **1** for monatin as no such coupling is possible for the lactone derived from the alternative structure **2**.

The relative configuration of the C-2 and C-4 chiral centres in monatin **1** was deduced from the (^{1}H, ^{1}H) NOE connectivity pattern established for the lactone **3** in a number of homonuclear ^{1}H{^{1}H} NOE experiments. Irradiation at the resonance position of one of the C-12 methylene protons (δ_H 2.775, J 10.3 and 13.2 Hz) resulted in an NOE only for the other C-12 methylene proton at δ_H 3.195 (J 9.2 and 13.1 Hz). In contrast, irradiation of the δ_H 3.195 resonance resulted in the observation of NOEs for 12-H (δ_H 2.775), 13-H (δ_H 3.916) and both C-10 methylene protons (δ_H 3.513 and 3.599). The implication of these results is that the C-13 proton and the indolylmethyl group at C-11 are located on the same face of the lactone ring, *i.e.* are *cis* orientated. Consequently, the lactone **3** must have the 11*R**,13*R** configuration and thus monatin **1** the 2*R**,4*R** configuration.

The 2*S* configuration of monatin followed from the molecular rotations calculated from the observed specific rotations of $[\alpha]_D^{20}$ −49.6 (H_2O) and −7.6 (HCl; 1 mol dm^{-3}). This finding is in line with the Clough–Lutz–Jirgenson rule[15–17] if it is assumed that the contribution of the C-4 centre to the molecular rotation is not influenced by the change in solvents. This assumption is justified as shown by the literature values for the contributions of the C-2 and C-4 centres to the molecular rotation calculated[18,19] from the observed specific rotations for (2*S*,4*R*)-{$[\alpha]_D^{20}$ −30.3 (H_2O); −8.3 (HCl; 0.2 mol dm^{-3})} and (2*S*,4*S*)-4-hydroxy-4-methylglutamic acid {$[\alpha]_D^{20}$ +0.5 (H_2O); +23.2 (HCl; 0.2 mol dm^{-3})} isolated from natural sources.[19–21] This result in conjunction with the known relative configuration (see above), establishes the 2*S*,4*S* configuration for monatin.

The sweetness of monatin salt was determined by an experienced ten-member taste panel trained according to established procedures.[22] Panel members were selected for their ability to detect and evaluate sweetness at recognition threshold values, *i.e.* the concentration at which the sensation of sweetness is just discernible. The panel determined the recognition threshold value of sucrose as 0.22–0.44% (w/v) which compares well with the accepted value of 0.3% (w/v). The monatin salt's relative sweetness at a recognition threshold value of 2.75 × 10^{-4}% (w/v) was determined as 800 times that of sucrose (calculated on the lowest value found for sucrose). In addition the panel found that monatin salt exhibited a very slight liquorice aftertaste. Subsequently the relative sweetness of the monatin salt was established as 1400 and 1200 times that of a 5 and 10% (w/v) sucrose solution, respectively. These findings make monatin a very attractive prospect as a high intensity sweetener.[23]

Experimental

M.p.s were determined on a Reichert Koffler hostage apparatus and are uncorrected. UV absorptions were measured on a Unicam SP8-100 spectrometer. Infrared spectra were recorded on a Beckman Acculab 8 spectrometer as KBr discs or for solutions in chloroform. Mass spectra were recorded on a Varian MAT 212 double focussing mass spectrometer. NMR spectra were recorded on a Bruker WM-500 (11.7 T) or AC-300 (7.0 T) spectrometers. Optical rotations, measured at 20 °C on a Perkin-Elmer 241 polarimeter, are recorded in units of 10^{-1} deg cm^2 g^{-1}.

Isolation of Monatin **1**.—Roots of *Schlerochiton ilicifolius* (160 kg) were collected in the vicinity of the Waterberge in the north-western Transvaal, air-dried and milled to a coarse powder (83 kg). Portions of the ground material (10 kg) were stirred overnight with water (25 dm^3) and the resulting slurry pressed in a mechanical press to obtain an aqueous extract which was filtered to remove fine particles. The extract was stirred in portions (2 dm^3) with Bio-Rad cation exchange resin (AG50WX-8, 500 cm^3) in the H$^+$-form. The resin was washed with water and the basic components removed from the resin by stirring with an ammonia solution (5%; 1 dm^3). The combined ammonia extracts were freeze-dried to give the crude basic components (658 g). The basic components were dissolved in water (2 dm^3) and portions (50 cm^3) were applied to a BioGel P2 gel filtration column (100 × 6 cm) and eluted with water at a flow rate of 5 cm^3 min^{-1}. The sweet-tasting fractions were combined and freeze-dried to give a crude product (143 g). Portions (4.0 g) of this product were applied to a Sephadex G10 gel filtration column (200 × 2.2 cm) and eluted with water at a flow rate of 2 cm^3 min^{-1}. Once again the sweet tasting fractions were combined and freeze-dried to give an impure sweet product (9.4 g). This product was again applied to a Sephadex G10 column but in smaller portions (300 mg) and eluted with water at the same flow rate as previously. The combined sweet tasting fractions were combined and freeze-dried to give *monatin* **1** (1.73 g) as a mixture of salts, $[\alpha]_D^{20}$ −49.6 (*c* 1.00 in H_2O), in which the sodium salt predominated (>95%).

The free amino acid was obtained as follows. A mixture of the monatin salts (100 mg) was dissolved in water (1 cm^3) and glacial acetic acid (1 cm^3) was added. Ethanol (96%; 5 cm^3) was then added and upon storage overnight at room temperature, the mixture gave fine rosettes of needle-like crystals of *monatin* or (2S,4S)-4-*hydroxy*-4-(*indol*-3-*ylmethyl*)*glutamic acid* **1**, m.p. 216–220 °C (gas evolution), $[\alpha]_D$−7.6 [*c* 1.00 in HCl (1 mol dm^{-3})]; λ_{max}[NaOH (1 mol dm^{-3})]/nm 279 (ε 5355); ν_{max}(KBr)/cm^{-1} 3396 (NH_2), 3020, 1580 (CO_2H) and 1540 (Found: C, 56.6; H, 5.5; N, 9.3. $C_{14}H_{16}N_2O_5 \cdot \frac{1}{4}H_2O$ requires C, 56.65; H, 5.6; N, 9.4%).

Methyl (2S,4S)-2-(*Indol*-3-*ylmethyl*)-4-(2,4-*dinitroanilino*)-5-*oxo*-2,3,4,5-*tetrahydrofuran*-2-*carboxylate* **3**.—Monatin **1** (60 mg) and sodium hydrogen carbonate (20 mg) were stirred for 10 min in an ethanol–water mixture (1:1 v/v). A solution of 1-fluoro-2,4-dinitrobenzene (200 mg) in ethanol (1 cm^3) was added and the mixture stirred overnight at room temperature. It was then acidified (6 mol dm^{-3} HCl) to pH 2 and extracted twice with diethyl ether. The ether extract was treated with an ether solution of diazomethane [generated from Diazald® (*N*-methyl-*N*-nitrosotoluene-*p*-sulfonamide) (2.14 g)] and after 30 min the excess of diazomethane was decomposed by the addition of a few drops of acetic acid. The ether was evaporated

in a stream of nitrogen and the crude product purified by column chromatography on silica gel using ethyl acetate–hexane (1:3 v/v) as eluent. Crystallisation from ethyl acetate–hexane yielded crystals of the *title lactone* **3** (25 mg), m.p. 174–175 °C, $[\alpha]_D^{20}$ +0.2 (*c* 1.00 in $CHCl_3$); $\nu_{max}(CHCl_3)/cm^{-1}$ 3350 (NH), 1788 (lactone) and 1740 (ester) (Found: C, 55.7; H, 4.0; N, 11.9%; M^+, 454.1114. $C_{21}H_{18}N_4O_8$ requires C, 55.5; H, 4.0; N, 12.3%; *M*, 454.1115).

Taste Evaluation of Monatin Salt.—Water used to dissolve the compounds in the taste evaluation was doubly distilled and then passed through a series of Millipore deionising filters. The sweetness of the compounds was assessed on a weight by weight basis. The taste panel consisted of 10 members with taste evaluation experience of foodstuffs and beverages, who were selected for their ability to recognise sweetness at the accepted threshold value for sucrose (0.3% w/v).

The recognition threshold value of sucrose was determined by the panel by tasting blind, sucrose solutions with the following concentrations ($\times 10^{-3}$): 0.2, 0.4, 0.8, 1.6, 3.2, 6.4 and 12.8 mol dm^{-3}. Panel members had to indicate at which concentration sweetness could be recognised. This test was repeated 10 times to ascertain the accuracy of the results. Analysis of the results indicated that the panel could detect sweetness in the 6.4–12.8 $\times 10^{-3}$ mol dm^{-3} range which corresponds to a recognition threshold value of 0.22–0.44% (w/v). Following this procedure the recognition threshold value of the monatin salt was determined as 2.75 $\times 10^{-4}$% (w/v) by tasting solutions over a concentration range of 0.2–0.35 mg/100 cm^3.

The relative sweetness of the monatin salt compared to a 5% and a 10% solution (w/v) of sucrose was determined by tasting solutions of the compound at different concentrations and selecting the concentration at which the taste closest approximated that of the sucrose solution. From the known concentration of the selected sample of monatin salt, the relative sweetness of monatin was determined as 1400 and 1200 times that of sucrose, respectively.

Acknowledgements

The authors thank the Foundation for Research Development, Pretoria for financial support (to R. V.).

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Paper 2/03485I
Received 1*st July* 1992
Accepted 17*th August* 1992