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Photostability study of natural high-potency sweetener monatin in a model beverage system and characterisation of the degradation products

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ABSTRACT

Photodegradation of the naturally occurring high-potency sweetener monatin was studied in a lemon–lime beverage model system under simulated conditions. Most of the monatin disappeared after treatment with the equivalent of four days of ultraviolet light exposure and resulted in the formation of a number of degradation products. These degradation products have been isolated and characterised in the present study. On the basis of these identified structures, a photodegradation pathway has been proposed suggesting that monatin gets oxidised on the indole C-2 position to result in 2-hydroxymonatin. It also undergoes decarboxylation resulting in a 4-oxopentanoic acid analog and a major rearrangement resulting in an isonicotinic acid analog. Monatin also degrades into 3-formylindole and indole-3-carboxylic acid. Besides these, a partial monatin dimer and a monatin lactone were identified as additional degradation products. Details of the isolation and characterisation are given.

1. Introduction

Natural high-potency sweeteners can be used in beverages to produce all natural low calorie products. Monatin is one such naturally occurring high-potency sweetener, an amino acid derivative which has been isolated from the root bark of a spiny-leafed hardwood shrub called *Sclerochiton ilicifolius*. This plant naturally grows in the northwestern Transvaal region of South Africa. Monatin has two chiral centres leading to four potential stereoisomers; (*2R*, *4R*) monatin, (*2S*, *4S*) monatin, (*2R*, *4S*) monatin, and (*2S*, *4R*) monatin. The first structure elucidation of isolated natural monatin reported it to be (*2S*, *4S*)-2-amino-4-carboxy-4-hydroxy-5-(3-indolyl)-pentanoic acid (Vleggaar, Ackerman, & Steyn, 1992). Lately, all four isomeric forms of monatin have been reported to occur naturally in this plant from different regions (Bassoli, Borgonovo, Busnelli, Morini, & Drew, 2005). Interestingly, these stereoisomers have been found to have different sweetening characteristics. It has been reported that the sweetness intensity is dependent on the optical purity of each stereoisomer, and (*2R*, *4R*) monatin is the sweetest among the four optical isomers, being about 2700 times sweeter in comparison to 5% (wt./vol) sugar (Amino & Hirasawa, 2005).

Monatin is present in only trace amounts, up to 0.007% by mass in the root bark of *S. ilicifolius*; therefore, it is not a source of potential commercial supply for monatin. There are numerous reports in the literature regarding the synthesis of monatin *via* various chemical (Nakamura, Baker, & Goodman, 2000) and chemoenzymatic routes (Buddoo et al., 2009). Monatin is being developed by Cargill (Brady, Steenkamp, Rousseau, Buddoo, & Steenkamp, 2009) and Ajinomoto (Amino & Hirasawa, 2006). Development of a sweetener requires hydrolytic, photo and thermal stability studies to assure viability of the sweetener. However there are no such stability study reports on monatin in the literature. Recently it has been reported that degraded monatin solution have presence of "musty" off flavours which is characteristic of 3-methyl indole (Skatole) and samples get discoloured after exposure to UV light (Evans & Goulson, 2010). Stability of monatin is thus an issue as it tends to degrade in solution at high temperatures and upon ultraviolet (UV) exposure especially under low pH conditions. There are few reports on the stabilisation of monatin in beverage formulations where photodegradation of monatin gets reduced with the help of added antioxidants (Roy, 2009), radical scavengers (Evans & Goulson, 2010) and with use of a UV-light shielding packaging (Mori, 2006). We have an interest in studying the stability of natural sweeteners under our product matrix and storage conditions. The present work is focused on studying in detail the photodegradation of monatin under UV irradiation and high temperature conditions in a lemon–lime beverage model system. From the degraded monatin containing beverage, seven of the resulting degradation products of monatin, compounds **1**–**7**, were isolated and characterised in order to understand monatin's degradation pathway.

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2. Materials and methods

2.1. Reagents and chemicals

2.1.1. (2R, 4R)-monatin

For this study we obtained monatin sample from in-house chemical synthesis of (*2R*, *4R*) monatin in high optical purity (+99%) using published procedures. Starting from indole first a mixture of the *R,R*/*S,S* stereoisomers of monatin was obtained following published procedures (Holzapfel, Bischofberger, & Olivier, 1994). This mixture was subjected to optical resolution via repeated optical salt formation steps as described in a 2005 patent application (Amino & Hirasawa, 2005). The enantiomeric purification was followed by chiral HPLC to confirm the stereo-isomeric enrichment (Buddoo, Rousseau, & Gordon, 2009).

2.1.2. Monatin lactam derivative

It was prepared for direct comparison with one of the degradation product, compound **2**. A sample of monatin was heated in aqueous solution for several hours at 60 °C following a published procedure (Ikeda, Kogiso, & Nakamura, 2007).

3-Formylindole and indole-3-caboxylic acid commercial samples for authentication and ammonium formate were purchased from Sigma–Aldrich (St. Louis, MO, USA). Acetonitrile used was HPLC grade. Water was purified using a Millipore system (Billerica, MA, USA).

Lemon–lime beverage samples at pH 2.6 without carbonation were prepared as reported earlier (Clos, DuBois, & Prakash, 2008).

2.2. Analytical instruments and conditions

2.2.1. LC–MS

Experiments were carried out on a Sciex API 150 single-quadrupole LC–MS system (AB Sciex, Framingham, MA, USA) with APCI and ESI sources running on the Analyst 4.1 platform. The LC–MS system had Agilent 1100 pumps and diode array detector and utilised a Gilson 215 autosampler. The LC–MS method employed Synergi Hydro-RP column (250 × 4.6 mm, 4 μm, p/n 00G-4375-E0; Phenomenex, Torrance, CA, USA) with UV detection at 280 nm and a binary solvent mobile phase (A, water with 50 mM ammonium formate, pH 4.0; B, acetonitrile) using a gradient (12% B for 5 min, 12–34% B over 5 min, 34–95% B over 5 min, 95% B for 5 min, 95–12% B over 1.0 min, 12% B for 9.0 min) at a flow rate of 1 mL/min. The injection volume was 200 μL. For compound **5**, solvent phase A was modified to have only 5 mM ammonium formate at pH 4.0.

2.2.2. HPLC

Waters 600 series semi-preparative HPLC system (Waters Corporation, Milford, MA, USA) was used with UV Detection, diode array detector (190–400 nm) monitored at 280 nm, running on the Empower software platform. HPLC method I employed Synergi Hydro-RP column (250 × 10 mm, 4 μm, p/n 00G-4375-N0, Phenomenex, Torrance, CA, USA) with a binary solvent mobile phase (A, water with 5 mM ammonium formate, pH 4.0; B, acetonitrile) using gradient (12% B for 5 min, 12–34% B over 5 min, 34–95% B over 10 min, 95% B for 5 min, 95–12% B over 0.1 min, 12% B for 4.9 min) at a flow rate of 5 mL/min. The injection volume was 100–400 μL.

HPLC method II employed Atlantis T3 C_{18} column (250 × 10 mm, 5 μm, p/n 1860,03,694, Waters Corporation, Milford, MA, USA) and a binary solvent mobile phase (A, water with 0.05% trifluoroacetic acid (TFA); B, acetonitrile with 0.05% TFA) using a gradient (0% B to 50% B over 30 min, 50–100% B over 1 min, 100–0% B over 1 min, 0% B for 9 min) at a flow rate of 5 mL/min. The injection volume was 500 μL.

cvvHPLC method III employed Atlantis dC_{18} column (250 × 4.6 mm, 5 μm, p/n 186003748, Waters Corporation, Milford, MA, USA) and a binary solvent mobile phase (A, water with 0.05% TFA; B, acetonitrile with 0.05% TFA) using a gradient (12% B to 50% B over 30 min, 50–95% B over 5 min, 95% B for 5 min, 95–12% B over 1 min, 12% B for 9 min) at 5 mL/min flow rate. The injection volume was 500 μL.

2.2.3. High resolution mass spectra

These were recorded using a Waters Premier Q-T of Mass Spectrometer (Waters Corporation, Milford, MA) running on the MassLynx software platform, version 4.1. Samples were prepared in a buffer consisting of H_2O-acetonitrile (1:1, v/v) + 0.1% formic acid and infused at a rate of 5 μL/min. MS parameters were as follows: Capillary voltages, 3.5 kV (ESI^+) or 2.5 kV (ESI^-); cone voltages, 35 V (ESI^+ and ESI^-) default, adjusted to provide ⩽0.1 ions per push (IPP); source temperature, 80 °C; desolvation temperature, 200 °C; desolvation gas flow 200 L/h; scanning from *m*/*z* 100–1200.

2.2.4. NMR

^1H and 2D NMR experiments were performed on a 500 MHz, Bruker Avance DRX NMR (Bruker BioSpin Corporation, Billerica, MA, USA) gradient system, running on the Xwin NMR software platform. Various probes were used including a 5 mm inverse detected z-gradient probe and a capillary NMR probe with a 10 μL flow cell.

2.3. Photodegradation experiments in a sunlight chamber

An Atlas Sun-test chamber (Atlas Material testing technology GmbH, Linsengericht, Germany) was used for simulating sun-light exposure to beverage samples. The programme used was a 6 h period of UV exposure in one run. Such a run is equivalent to one day of sun-light exposure in Arizona or 400 langley at 40 °C. Here langley is defined as, "a unit of energy per unit area, equal to 1 gram-calorie/cm^2 commonly employed in radiation theory" (Weast, 1983).

2.4. Monatin degradation and mass balance analysis

First a small scale degradation of monatin was carried out under UV irradiation at 40 °C in a lemon–lime beverage model system at 30 μg/mL in the Atlas sun-test chamber using regular transparent polyethylene terephthalate (PET) bottles. The total UV exposure was carried out for 24 h (equivalent to four days of sun-light exposure in Arizona). Black PET bottles were used as a control to standardise conditions. The degraded monatin solution was analysed in triplicate using LC–MS. The average peak area was used to determine the calculated concentration using the response factor determined for monatin. The calculated concentrations assume that the degradation products have the same response factor as monatin.

A large scale UV-degradation was carried out at the 50 μg/mL level in lemon-lime matrix using 300 mL size transparent PET bottles. The calculated concentration of monatin remaining after degradation was found to be approximately 20%. The combined relative abundance for monatin and the peaks selected for isolation and characterisation in this study was 89.2%. This suggested that either the response factor varied significantly between monatin and one or more of the degradation products or some portion of the degraded monatin was not observed as integrated peaks within the selected chromatographic window. The degraded monatin samples showed a precipitate that settled out on the bottom of the bottles after they were left undisturbed at 4 °C for several days.

This precipitate may account for some portion of the degraded monatin that was not observed in the UV chromatogram.

2.5. SPE (solid phase extraction) concentration and degradant isolation

The degraded monatin solution was subjected to SPE concentration step to provide a smaller volume of concentrated sample that was largely free of acid and unretained components. One 300 mL bottle of degraded monatin solution was passed through a 5 g C_{18} SPE cartridge that had been washed with 3 column volumes of methanol and equilibrated with three column volumes of H_2O. After addition of the degraded monatin solution, the SPE cartridge was washed with 3 column volumes of H_2O and then eluted with 3 column volumes of methanol. The methanol layer was collected and concentrated to dryness by rotary evaporation under reduced pressure. The resulting concentrated degradant fraction was taken up in 1.0 mL of H_2O for subsequent HPLC fractionation. This procedure was repeated for each bottle of monatin solution.

Preliminary HPLC fractionation was carried out following method I. The individual degradation product peaks were pooled from multiple runs to yield the crude impurity fractions. Preliminary analysis indicated that the crude impurity fraction containing compound **1** required additional separation. Therefore, a second round of chromatography using HPLC method II was undertaken to further purify the sample. However, it resulted in two peaks eluting at 14.3 and 15.3 min and both had the same $[M + H]^+$ ion as expected for compound **1**. Furthermore, re-injection of either peak resulted in a mixture of both peaks. This suggested that **1** exists as a mixture of isomers that readily interconvert. Therefore both peaks (14.3 and 15.3 min) were collected, combined and concentrated for further analysis.

Compounds **2**, **3**, and **7** isolated from the preliminary HPLC fractionation were found to be of sufficient purity for structural characterisation and so were used without further purification. Crude impurity fractions containing compounds **4** and **6** required an additional purification and a second round of chromatography was undertaken using HPLC method III to further purify sufficient samples for structural characterisation.

Various attempts to purify the crude impurity fraction containing compound **5** via HPLC were not very successful and the purified material was always found to contain multiple components resulting from the apparent decomposition of **5**. An attempt was made to isolate **5** from the crude fraction using the LC–MS method and the modified solvent phase A (5 mM ammonium formate, pH 4.0). NMR analysis of **5** thus obtained did not produce any useful data and only MS/MS fragmentation was obtained.

3. Results and discussion

3.1. Photodegradation of monatin

Monatin in regular transparent PET bottles degraded by 70% in just one day's equivalent of UV exposure while it was completely gone by four days equivalent of UV exposure. However, in the dark PET bottle which was our control in the sun-test chamber, monatin was stable even up to 92% after four days of UV exposure showing that blocking UV light was useful in stabilising monatin. Under visible light (regular laboratory light), monatin degraded only very little and was present up to 90% in normal as well as dark PET bottles (Fig. 1).

It was observed that samples in regular PET packaging after UV exposure changed colour, from colourless in the beginning to yellow after exposure. A group of trained in-house expert flavourists carried out odour analysis of the degraded beverage samples and confirmed that the samples before exposure had no malodor but after exposure had presence of a strong musty/fecal like odour which was hard to miss. This result was the same as described previously (Evans & Goulson, 2010) where degraded monatin solution was reported to have the presence of 3-methyl indole (Skatole) which has a characteristic musty/fecal like odour.

3.2. Identification of the degradation products of monatin

The sample of degraded monatin beverage when concentrated via SPE generated useful MS data from the LC–MS analysis (Table 1). A sample of the base matrix used was also analysed under similar conditions to exclude peaks which were already present in the base matrix from further analysis (Fig. 2A). Under this method, the monatin peak was observed to elute at 6.42 min (Fig. 2B). A number of potential monatin degradation product peaks were observed to elute between 2 and 17 min in the UV chromatogram (Fig. 2C). The nine largest peaks, including monatin, which could not be attributed definitively to the base matrix, are thus labelled in Fig. 2C. One peak observed at 10.25 min was not observed right after degradation but grew overtime as the samples were stored at 4 °C and so was not included in the analysis. Out of these nine peaks seven of the resulting degradation products of monatin, compounds **1–7** (peaks 1–7), were isolated and characterised using a combination of MS, MS–MS and 1D and 2D NMR techniques.

In order to have a reference for structural characterisation of the isolated degradants, a detailed NMR dataset including ^1H NMR, ^{13}C NMR, ^1H–^1H COSY, HSQC and HMBC spectra, was acquired for monatin. Accurate mass analysis using HRESI$^+$ gave an $[M + H]^+$ ion at *m/z* 293.1142 and the fragmentation pattern was acquired for this ion by ESI + TOF MS/MS. All these assignments were found to be in agreement with data reported in literature (Bassoli et al., 2005; Holzapfel et al., 1994).

3.2.1. Identification of the degradation product, compound *1*

The molecular formula of compound **1** was deduced to be $C_{14}H_{16}N_2O_6$ on the basis of the ESI + LC–MS analysis which showed an $[M + H]^+$ ion at *m/z* 309 along with a fragment ion at *m/z* 291 $[M + H–H_2O]^+$. The $[M + H]^+$ ion was observed at *m/z* 309.1093 in the HRESI + TOF data which indicated that the mass of the $[M + H]^+$ ion was in good agreement with the molecular formula (calculated for $C_{14}H_{17}N_2O_6$: 309.1087, error: 1.9 ppm). This molecular formula contained the net addition of one oxygen atom relative to monatin.

The fragmentation pattern acquired by ESI + TOF MS/MS selecting the $[M + H]^+$ ion at *m/z* 309.0 for fragmentation was similar to that observed for monatin. A notable difference from the monatin fragmentation was observed for the fragment ions attributable to the indole moiety. A pair of fragment ions corresponding to the indole moiety was observed at *m/z* 134 and 146. The molecular formula for the ion at *m/z* 146 contained one additional oxygen atom relative to the ion observed at *m/z* 130 for monatin. These data indicated that the additional oxygen atom present in compound **1** must be located within the indole moiety. This was further supported by NMR analysis (Table 2).

The ^1H NMR of **1** confirmed the presence of two isomers which resulted in the appearance of two sets of signals in the NMR data. One set of signals was of somewhat higher relative abundance and was designated as the major isomer. For compound **1** (major), the α-methine proton H-2 was assigned to a multiplet at 3.66 ppm. Methylene protons at H-3 were assigned to a doublet of doublets at 1.82 ppm (J = 7.3, 14 Hz) and a multiplet at 2.07 ppm. Methylene protons at H-5 were observed as a multiplet at 2.08 and a doublet at 2.30 ppm (J = 14.6 Hz). COSY correlations were observed between the H-2/H-3 protons while the H-5 protons did not show any additional COSY correlations. Assignment of the indole region between 6.77–7.39 ppm by ^1H NMR and COSY correlations (H4′/

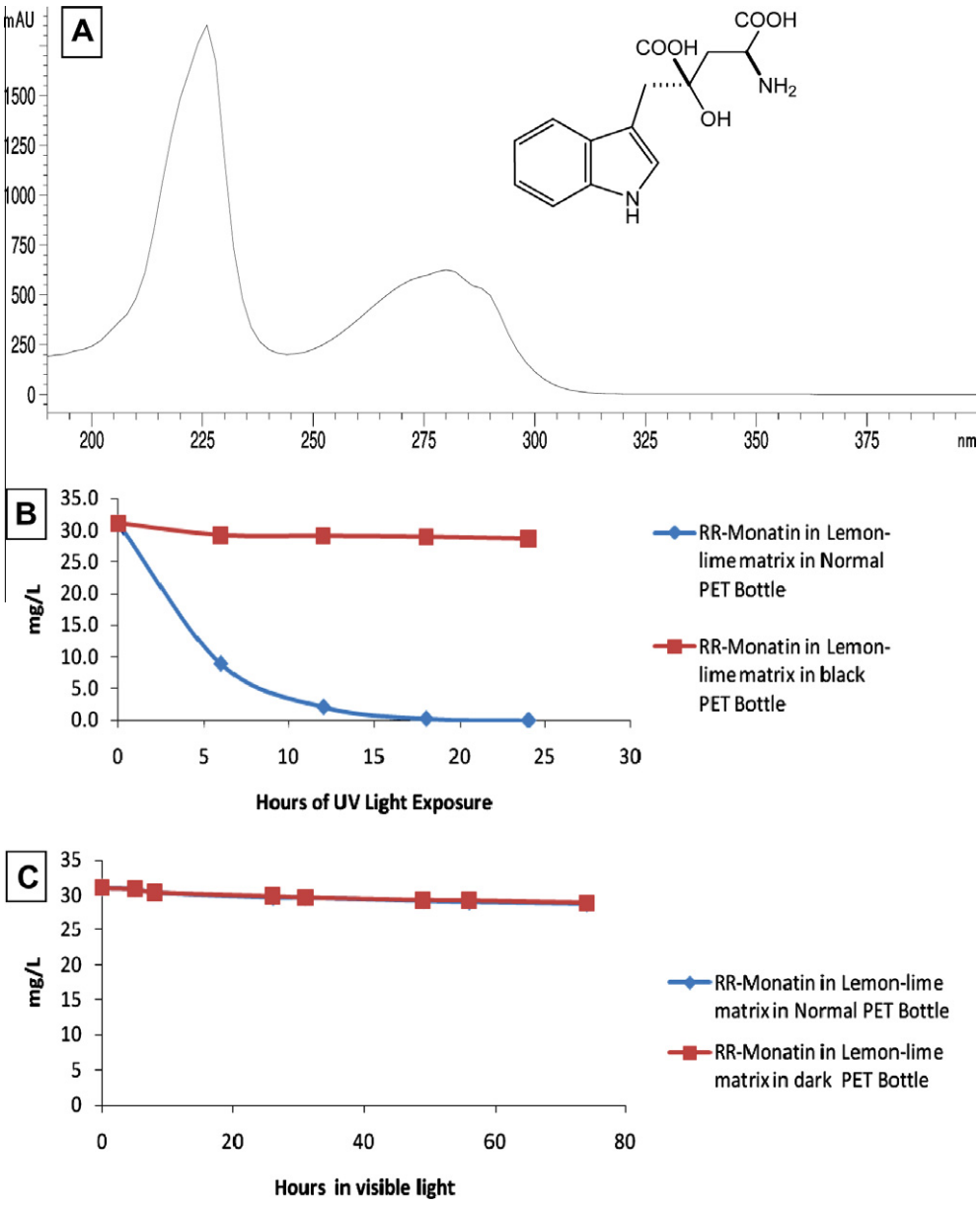

Fig. 1. (A) (*2R*, *4R*) Monatin and its UV–vis spectrum. (B and C) Concentration of monatin as a function of time in regular PET and black PET bottles under UV light and visible light.

H5′, H5′/H6′, H6′/H7′) was straightforward. The singlet corresponding to H-2′, observed at 7.12 ppm for monatin, was clearly absent from the ^{1}H spectrum (and HSQC data) acquired for **1**. In addition, the indole NH was observed as a singlet at 10.33 ppm and showed no correlation in the COSY spectrum. These data indicated that the addition of oxygen in **1** had occurred at C-2′ of the indole moiety resulting in the presence of a hydroxyl group at this position for the major isomer. The carbon resonances were attributed using two dimensional HSQC data. Carbons C-2, C-3, C-5, C-4′, C-5′, C6′ and C-7′ were assigned to 50.8, 40.2, 36.9, 125.0, 120.9, 127.0 and 108.5 ppm through their direct $^{1}H–^{13}C$ correlations with the corresponding protons.

The assignment of the minor isomer of **1** was made in a similar fashion. An additional COSY correlation was observed between the H-5 methylene protons at 1.92 ppm and a methine proton at 3.67 ppm which was assigned as H-3′ of the indole. This suggested that the position of the C-2/C-3 double bond in the indole moiety had shifted to yield the structure for the minor isomer illustrated in Fig. 3. Thus compound **1** was established as 2-hydroxy monatin which is present as a mixture of isomers depending on the position of the unsaturation (Fig. 3).

3.2.2. Identification of the degradation product, compound *2*

The molecular formula of compound **2** was deduced to be $C_{14}H_{14}N_2O_4$ on the basis of ESI + LC–MS analysis which showed an $[M + H]^+$ ion at *m/z* 275 and an $[M + H]^+$ ion at *m/z* 275.1044 in the HRESI + TOF mass spectrum. The molecular formula confirmed that it has a structure resulting from the net loss of H_2O relative to monatin. Monatin is known to exist in equilibrium with its lactam and lactone derivatives. Therefore, **2** could likely be either of these 2 compounds. The fragmentation pattern for this ion obtained by ESI + TOF MS/MS showed a notable difference from the fragmentation pattern for monatin in the absence of a fragment ion corresponding to loss of one or more H_2O units. This suggested that **2** may not have a free hydroxyl group and so could correspond to the lactone (ester) of monatin which lacks a free hydroxyl group at C-4. In contrast, monatin lactam retains the hydroxyl group at C-4 and so would be expected to show loss of H_2O. Fragment ions at

Table 1
HPLC, MS and MS/MS data of monatin and its degradation products.

Peak	R_t (min)	$m/z(ES^+)$			$m/z(ES^-)$		Chemical formulae	Compound
		$[M+H]^+$	$[M+Na]^+$	Fragments	$[M-H]^-$	Fragments		
1	4.12	309		291, 273, 263, 246, 228, 146, 134			$C_{14}H_{16}N_2O_6$	2-Hydroxy monatin
	6.42	293		275, 257, 247, 230, 212, 158, 130, 132			$C_{14}H_{17}N_2O_5$	Monatin
2	7.55	275		229, 212, 183, 168, 158, 118			$C_{14}H_{14}N_2O_4$	Monatin lactone
3	10.51	247		201, 230, 212, 184, 174, 158, 132, 118			$C_{13}H_{14}N_2O_3$	(R)-2-Amino-5-(1H-indol-3-yl)-4-oxopentanoic acid
4	11.73	243		215	241	197, 179, 169, 118	$C_{13}H_{10}N_2O_3$	2-(2-Formamidophenyl) isonicotinic acid
5	12.61	535		517, 418, 400, 374, 293, 243,	533	515, 489, 471, 416, 372, 354	$C_{27}H_{26}N4O_8$	Unknown
6	14.41				160	116	$C_9H_7NO_2$	Indole-3-carboxylic acid
7	14.91	146		118	144		C_9H_7NO	3-Formyl-indole
8	16.12	n.d.						
9	16.31	199						

m/z 229.0982 $[M+H-COOH]^+$ and at *m/z* 212.0734 $[M+H-COOH-NH_3]^+$ suggested the presence of a free amine and further supported **2** to be the ester rather than the lactam. A sample of monatin lactam for direct comparison was prepared following a reported procedure (Ikeda et al., 2007) and its structure was confirmed in comparison with published ^1H NMR data. Analysis of the lactam sample by LC–MS indicated the presence of a peak with an $[M+H]^+$ ion at *m/z* 275 that elutes after **2** and is not observed as a significant peak in the degraded monatin sample confirming that **2** cannot be the lactam. Analysis of the ^1H NMR, COSY and HSQC data further supported compound **2** to be the monatin lactone derivative (Fig. 4).

3.2.3. Identification of the degradation product, compound *3*

The molecular formula of compound **3** was deduced to be $C_{13}H_{14}N_2O_3$ on the basis of ESI + LC–MS analysis which showed an $[M+H]^+$ ion at *m/z* 247 and HRESI$^+$ which displayed $[M+H]^+$, $[M+Na]^+$, and $[M+2Na-H]^+$ ions at *m/z* 247.1087, 269.0909, and 291.0728, respectively. Accurate mass analysis indicated that the mass of the $[M+H]^+$ ion was in good agreement with the molecular formula (calcd for $C_{13}H_{15}N_2O_3$: 247.1083, error: 1.6 ppm). Relative to monatin, this indicated a net loss of CH_2O_2 (46 Da) suggesting that one of the carboxylic acid groups present in monatin has been lost during the formation of this degradation product. The fragmentation pattern acquired by ESI + TOF MS/MS selecting the $[M+H]^+$ ion at *m/z* 247.0 for fragmentation showed fragment ions at *m/z* 201.1026, $[M+H-CH_2O_2]^+$, at *m/z* 184.0762 $[M+H-CH_2O_2-NH_3]^+$ and 230.0820 $[M+H-NH_3]^+$. The fragment ions at *m/z* 158.0598 and 174.0917 suggested that the degradant may lack the carboxylic acid group present at C-4 in monatin, but were not conclusive. The fragment ions at *m/z* 118.0958 and 132.0800 correspond to the indole moiety. The ^1H NMR data analysis (Table 3) of **3** indicated the presence of an α-methine proton at 3.81 ppm which was assigned as H-2. Two methylene protons at 3.00 and 3.21 ppm showing COSY correlation with H-2 were assigned as H-3 and seemed to be shifted downfield relative to monatin. This downfield shift could be due to the loss of the C-1 carboxyl group resulting in an unsaturation between C-2 and C-3, but this was not observed. Another, less likely, scenario would be that the carboxylic acid at C-1 has been lost with the additional unsaturation necessary to satisfy the molecular formula located elsewhere in the structure, but this would have resulted in a methylene group at C-2 rather than the observed α-methine. These data indicated that the carboxylic acid group at C-4 must have been lost during the formation of compound **3**. Two additional isolated methylene protons appeared as a broad singlet (3.90 ppm) and were assigned as H-5 which indicated that the unsaturation must be present at C-4 yielding a ketone at this position. Five protons between 7.01 and 7.49 ppm in ^1H NMR and COSY correlations (NH/H2′, H4′/H5′, H5′/H6′, H6′/H7′) were characteristic of the indole region indicating that it was unchanged in **3**. The carbon resonances were attributed using the HSQC data. Carbons C-2, C-3, C-5, C2′, C-4′, C-5′, C6′ and C-7′ were assigned to 51.5, 42.2, 40.5, 119.2, 120.1, 122.5 and 112.2 ppm through their direct $^1H-^{13}C$ correlations with the corresponding protons. Thus, **3** results from the loss of the carboxylic acid functionality at C-4 of monatin and has a ketone at this position. It was assigned to be 2-amino-5-(1H-indol-3-yl)-4-oxopentanoic acid (Fig. 3). We later found that this compound was reported before in a Japanese patent as a synthetic intermediate to monatin (Amino, Kawahara, Funakoshi, & Sugiyama, 2003). This is the first time reporting detail structural characterisation including COSY and HSQC NMR data on this compound.

3.2.4. Identification of the degradation product, compound *4*

The molecular formula of compound **4** was deduced to be $C_{13}H_{10}N_2O_3$ on the basis of ESI + LC–MS analysis which showed an $[M+H]^+$ ion at *m/z* 243 and HRESI$^+$ which displayed an $[M+H]^+$ ion at *m/z* 243.0771 which was in good agreement with the molecular formula (calculated for $C_{13}H_{11}N_2O_3$: 243.0770, error: 0.4 ppm). Relative to monatin this indicated a net loss of CH_6O_2. The ESI + TOF MS/MS fragmentation acquired by selecting the $[M+H]^+$ ion at *m/z* 243.0 was limited to a single fragment ion at *m/z* 215.0822 $[M+H-CO]^+$. Loss of CO was not observed before as a significant fragment ion for monatin or any of the degradants discussed previously. Fragmentation using negative mode ESI and selecting the $[M-H]^-$ ion at *m/z* 241.0 resulted in fragment ions at *m/z* 197.0714 $[M-H-CH_2O_2]^-$ suggesting that **4** has at least one carboxylic acid moiety and further losses led to ions at *m/z* 179.0608 $[M-H-CH_2O_2-H_2O]^-$ and 169.0763 $[M-H-CH_2O_2-CO]^-$.

Analysis of the ^1H NMR data (Table 3) indicated a lack of upfield signals and suggested a significant rearrangement of C-1 through C-5. Eight aromatic protons were observed in the ^1H NMR between 7.26 and 8.87 ppm. Four protons, two triplets at 7.26 (J = 7.9 and 7.45 ppm (J = 7.7), and two doublets at 7.73 (J = 7.9) and 8.24 ppm (J = 8.5 Hz) corresponded to an aromatic spin system and showed COSY correlations which were assigned to protons H-3′ to H-6′, respectively. The ^{13}C NMR values for all the carbons were assigned on the basis of HSQC and HMBC spectra. Analysis of the ^{13}C NMR data indicated that a ^{13}C signal around 110 ppm typical of the C-7 position of the indole moiety was absent suggesting that the indole group was absent or significantly modified. HMBC correlations between the aromatic singlet at 8.32 ppm to the quaternary carbon at 135.6 ppm and the presence of a signal at160.0 ppm in the ^{13}C

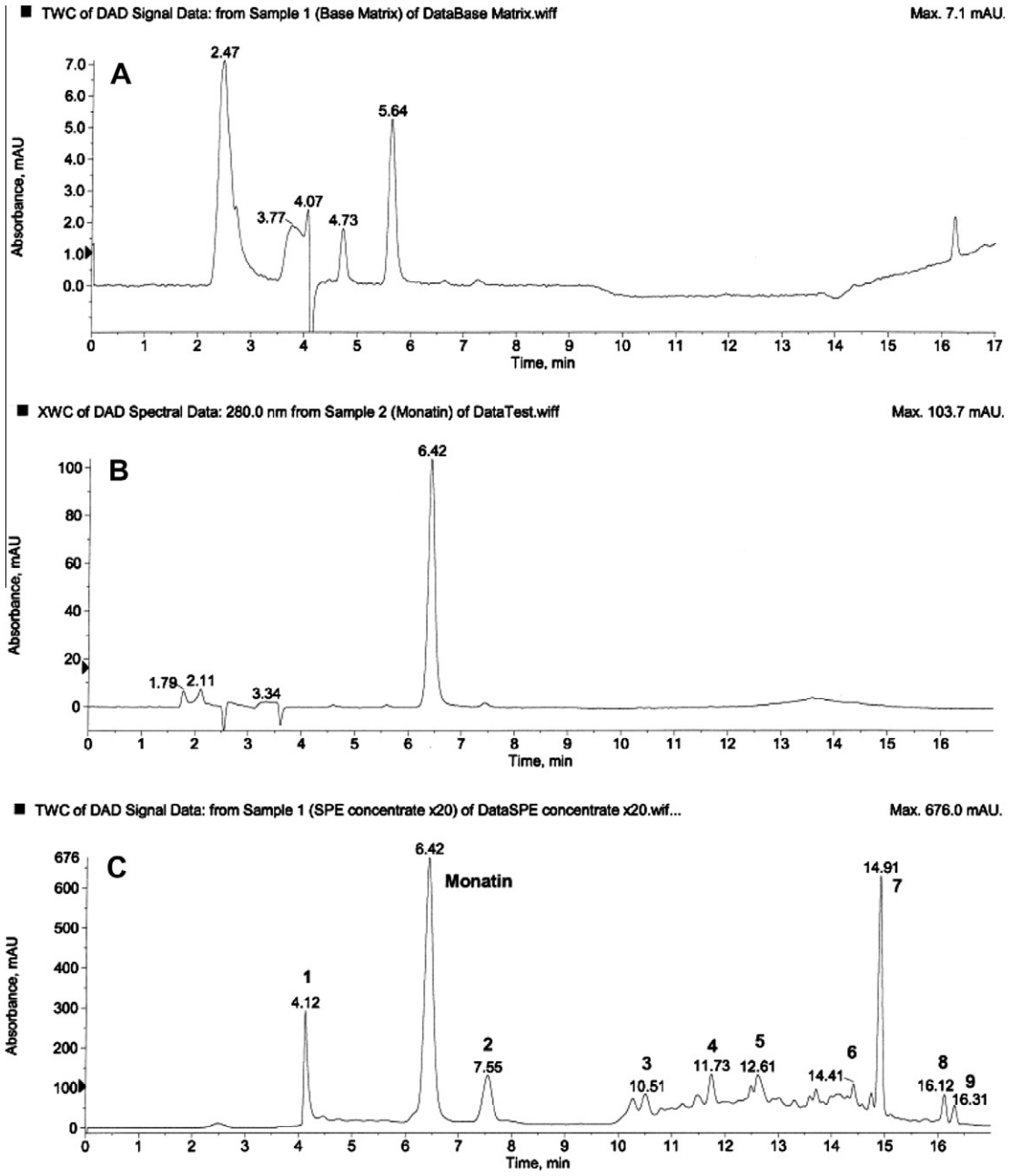

Fig. 2. (A) LC–MS analysis of the base matrix alone showing the UV (280 nm) chromatogram. (B) LC–MS analysis of monatin sample before degradation showing the UV (280 nm) chromatogram. (C) 20× SPE concentrated monatin degradant sample showing the UV (280 nm) chromatogram.

spectrum correlated to a singlet at 10.95 ppm suggested the presence of an *N*-formyl group. This was supported by the MS data above which showed loss of CO. These data suggested that the indole group had undergone ring opening between C-2′ and C-3′. This *N*-formyl phenyl ring was found to be attached to a substituted pyridine on the basis of HMBC correlations. HMBC correlations from the proton at 8.11 ppm to C-2 of the pyridine and the quaternary carbon at 127.9 ppm allowed it to be assigned as H-3 within the pyridine moiety. This proton also showed an additional HMBC correlation to a carbon at 166.0 ppm which was assigned as a carboxyl substituent at C-4. The remaining pair of aromatic doublets (J = 4.9 Hz) at 7.83 and 8.87 ppm showed a COSY correlation to each other and were assigned as H-5 and H-6, respectively. The various COSY and HMBC correlations thus identified were (H-3′/H-4′, H-4′/H-5′, H-5′/H-6′, H-5/H-6) and (CHO/C-2′, H-3′/C-1′, H-5′/C-1′, H-6′/C-2′, C-2, H-3/C-1′ ,C-2, COOH, H-5/C-3, COOH, H-6/C-2, C-4), respectively. Therefore, the structure of **4** was established as 2-(2-formamidophenyl) isonicotinic acid (Fig. 3).

3.2.5. Identification of the degradation product, compound *5*

The molecular formula for **5** was deduced to be $C_{27}H_{26}N_4O_8$ from the ESI + LC–MS analysis which an $[M + H]^+$ ion at *m/z* 535. The HRESI + TOF mass spectrum showed the accurate mass for the $[M + H]^+$ ion at *m/z* 535.1832 which was in good agreement with the molecular formula (calculated for $C_{27}H_{27}N_4O_8$: 535.1829, error: 0.6 ppm). Relative to monatin, this indicated a net addition of $C_{13}H_{10}N_2O_3$. The fragmentation pattern acquired by ESI + TOF MS/MS selecting the $[M + H]^+$ ion at *m/z* 535.0 for fragmentation resulted in a fragment ion observed at *m/z* 293.1167 which corresponded to monatin and suggested that one half of the structure is composed of monatin. The reciprocal fragment ion was observed at *m/z* 243.0804 and had the same molecular

Table 2
^{1}H and ^{13}C NMR data of the major and minor isomer of compound **1**.

Degradant position no.	Compound **1** major (DMSO-d_6)		Compound **1** minor (DMSO-d_6)	
	δ_H ^{1}H (ppm); *J* (Hz)	δ ^{13}C	δ_H ^{1}H (ppm); *J* (Hz)	δ ^{13}C
2-Amino-4-hydroxy pentanedioic acid residue				
1	–	–	–	–
2	3.66; m	50.8	3.31; d (7.3)	41.8
3	1.82; dd (7.3, 14.0) 2.07; m	40.2	1.65; dd (7.3, 14.0) 2.36; m	40.8
4	–	–	–	–
5	2.08; m 2.30; d (14.6)	36.9	1.92; dd (12.2, 15.3) 2.34; m	37.8
Indole moiety				
1′	10.33; s	–	10.26; s	–
2′	–	–	–	–
3′	–	–	3.67; m	40.5
4′	7.39; d (7.3)	125.0	7.58; d (7.3)	126.1
5′	6.88; t (7.3)	120.9	6.88; t (7.3)	120.9
6′	7.12; t (7.3)	127.0	7.10; t (6.7)	127.0
7′	6.77; d (7.9)	108.5	6.74; d (7.9)	108.4
3a′	–	–	–	–
7a′	–	–	–	–

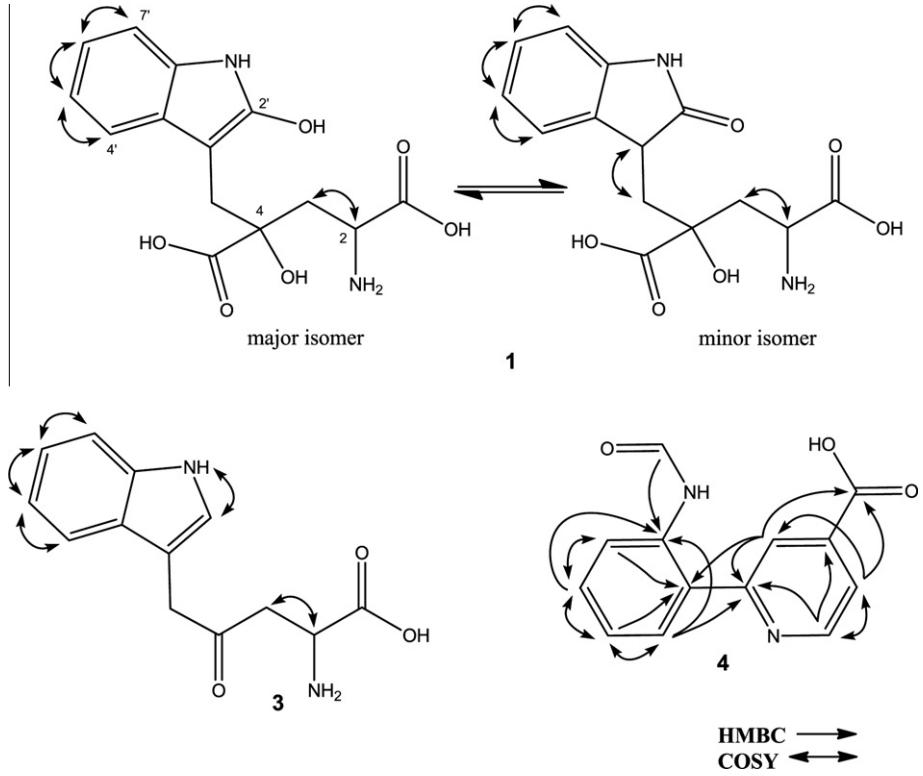

Fig. 3. Compounds **1**, **3** and **4** and their key COSY and HMBC correlations.

formula as compound **4**. Fragment ions were also observed corresponding to loss of an indole moiety ion at *m/z* 418.1268 and net loss of an indole and CO_2 at *m/z* 374.1406. Loss of H_2O from the $[M + H]^+$ ion or the ion at *m/z* 418.1268 provided fragment ions at *m/z* 517.1716 and 400.1158, respectively.

The fragmentation pattern acquired by ESI-TOF MS/MS selecting the $[M - H]^-$ ion at *m/z* 533.0 resulted in fragment ions from several losses of H_2O and CH_2O_2 at *m/z* 515.1572, 489.1742, and 471.1662 besides fragment ions at *m/z* 416.1203 [M – H–indole moiety]$^-$, *m/z* 372.1216 [M – H–indole moiety–CO_2]$^-$ and with further loss of H_2O yielding a fragment ion at *m/z* 354.1101. No fragments corresponding to two halves were seen as was the case in positive mode fragmentation.

Due to the limited stability of this degradant, NMR data were not of use for assigning the structure. Thus, compound **5** is proposed to have a structure which is a partial dimer of monatin with one half of the structure composed of a unit of monatin and the second half having the same molecular formula as compound **4**. We hypothesise that the monatin moiety is likely attached through the amine at C-2 as represented in Fig. 4.

3.2.6. Identification of the degradation product, compound *6*

Compound **6** was given the molecular formula of $C_9H_7NO_2$ on the basis of ESI + LC–MS analysis which showed the $[M - H]^-$ ion at *m/z* 160 in the positive ion mode. Accurate mass from HRESI-TOF mass spectrum showed an $[M - H]^-$ ion at *m/z* 160.0392 (calculated for $C_9H_6NO_2$: 160.0399, error: −4.4 ppm). The fragmentation pattern acquired by ESI-TOF MS/MS selecting the $[M - H]^-$ ion at *m/z* 160.0 was dominated by a single fragment ion at *m/z* 116.0504 resulting from the loss of CO_2 and suggested the presence of a carboxylic acid functionality in the structure of the degradant. Analysis of the ^{1}H NMR data indicated a lack of upfield signals and

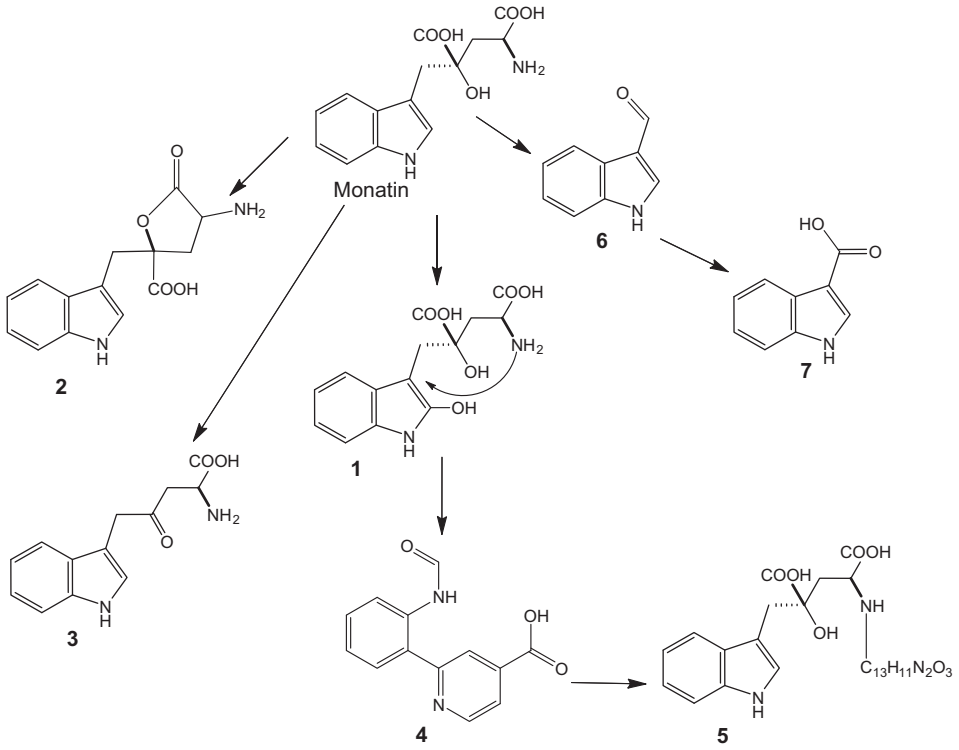

Fig. 4. Proposed monatin degradation pathway under UV conditions.

Table 3
^{1}H and ^{13}C NMR data of of compound **3** and **4**.

Degradant position	Compound **3** (CD_3OD)		Compound **4** (DMSO-d_6)	
	δ_H ^{1}H(ppm); J(Hz)	δ ^{13}C	δ_H ^{1}H(ppm); J(Hz)	δ ^{13}C
1			–	–
2	3.81; d (7.1)	51.5	–	158.0
3	3.00; dd (7.1, 18.5) 3.21; d (18.5)	42.2	8.11; s	122.0
4	–	–	–	139.5
5	3.90; s	40.5	7.83; d (4.9)	121.0
6			8.87; d (4.9)	149.5
C4-COOH			–	166.0
1′		–	–	127.9
2′	7.20; s	125.1	–	135.6
3′	–	–	8.24; d (8.5)	122.3
4′	7.49; d (7.8)	119.2	7.45; t (7.7)	129.5
5′	7.01; t (7.8)	120.1	7.26; t (7.9)	124.3
6′	7.09; t (7.6)	122.5	7.73; d (7.9)	129.7
NHCHO			10.95; s, 8.32; s	160.0
7′	7.34; d (7.8)	112.2		
3a′				
7a′				

suggested it to be a 3-substituted indole structure. A comparison with the commercially available compound (Sigma–Aldrich 284734, indole-3-carboxylic) provided a final confirmation of the structure. The ^{1}H NMR spectrum for the commercial standard was found to be identical to that of the isolated sample. LC–MS analysis indicated that compound **6** and the authentic sample of indole-3-carboxylic acid have the same retention time and give a single peak upon co-injection. Thus, the structure of compound **6** was assigned as indole-3-carboxylic acid.

3.2.7. Identification of the degradation product, compound **7**

Compound **7** was deduced to have a molecular formula C_9H_7NO based on the ESI + LC–MS analysis which showed the $[M + H]^+$ ion at *m/z* 146 in positive ion mode. Accurate mass analysis from HRESI + TOF mass spectrum showed an $[M + H]^+$ ion at *m/z* 146.0600 (calculated for C_9H_8NO: 146.0606, error: −4.1 ppm). The fragmentation pattern acquired by ESI + TOF MS/MS selecting the $[M + H]^+$ ion at *m/z* 146.0 for fragmentation was dominated by a single fragment ion at *m/z* 118.0584 $[M + H–CO]^+$ suggesting the presence of a formyl group in the structure of the degradant. ^{1}H NMR data indicated a lack of upfield signals and suggested a structure composed predominately of a 3-substituted indole similar to that observed for compound **6**. The presence of a formyl group was further confirmed by the presence of a singlet at 9.92 ppm in the ^{1}H spectrum which was correlated to an aldehyde carbon at 184.6 ppm in the HSQC spectrum. A comparison with the commercially available compound (Sigma–Aldrich 12944-5, 3-formylindole) was carried out. A ^{1}H NMR spectrum for the commercial standard was obtained and was found to be identical to that of our isolated sample. LC–MS analysis indicated that compound

7 and the authentic sample have the same retention time and give a single peak upon co-injection. The structure of **7** was thus assigned as 3-formylindole.

3.3. Sensory analysis on **6** and **7**

Out of all these degradants, in-house sensory analysis was carried out on commercially available 3-formyl indole and 3-carboxyl indole in aqueous solution. In literature (Tolonen et al., 2000) 3-formyl indole has been reported as a flavour enhancing compound. It was found that in 0.1% (wt./vol) solution, 3-formyl indole had Skatole like 'musty' smell same as was observed in the sensory analysis of the degraded monatin solution while 3-carboxyl indole had no such off flavour notes.

3.4. Possible mechanism and pathway for photodegradation of monatin

Monatin is known to exist in equilibrium with its lactam and lactone derivatives. However, in the present study no lactam derivative was found before or after the degradation. Only the lactone derivative was found to be present to some extent (~1%) in the monatin sample before degradation but increased in concentration to about 4% after degradation.

Thus under the present conditions of low pH in lemon–lime matrix (pH = 2.6) and under sun-simulated exposure, we found monatin degrading into seven main compounds. Compounds **1** and **4** were characterised fully as novel molecules but compound **5** was found to be unstable and could not be characterised completely. Major degradation products were compounds **1** and **7** with 13% and 6% of the original starting concentration of monatin, respectively. The possible pathway proposed for the degradation of monatin is based on the structural identities of the various degradation products that were obtained (Fig. 4). Oxidation of monatin resulted in formation of **1** first which probably undergoes significant rearrangement by opening of the indole ring between C-2′ and C-3′ and ultimately yielding an *N*-formyl group derived from C-2′. The substituted pyridine appears to result from bond formation between the monatin amine and C-3′ of the indole group. Compound **3** probably resulted from loss of CO and water from monatin. We speculate that compound **5** is probably the result of an addition of compound **4** to monatin through its amine at C-2 based upon our partial characterisation results. Monatin probably also photodegrades to give 3-formyl derivative. There are reports in the literature about 2, 3-substituted indole derivatives giving corresponding formyl derivatives upon photoxidation in acidic conditions (Mudry & Frasca, 1973). Therefore, monatin and even **2** and **3** may oxidise to **6** which can further oxidise to **7**.

4. Conclusion

In present work the degradation of monatin in a model lemon–lime beverage system has been studied to assess the viability of this sweetener in our product conditions. We have found that upon UV exposure monatin is unstable in a regular transparent PET bottle while it survived well in the control dark PET bottle. We have identified seven compounds as major degradation products of monatin. Structures could be unambiguously determined for six of the seven degradants. Two novel compounds were identified namely 2-hydroxy monatin and 2-(2-formamidophenyl) isonicotinic acid. A final structure for one of the degradation product has been only partially determined due to its instability during the purification. Two of the degradants 3-formyl indole and 3-carboxyl indole were confirmed by direct comparison with authentic commercial standards. Two remaining degradation products found were monatin lactone and 2-amino-5-(1H-indol-3-yl)-4-oxopentanoic acid. Sensory analysis of the commercially available samples of 3-formyl indole and 3-carboxyl indole indicated that former had Skatole like odour same as observed in the sensory analysis of the degraded monatin solution.

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