Monatin and Its Stereoisomers: Chemoenzymatic Synthesis and Taste Properties

Angela Bassoli,*[a] Gigliola Borgonovo,[a] Gilberto Busnelli,[a] Gabriella Morini,[a] and Michael G. B. Drew[b]

Dedicated to the memory of Professor Murray Goodman

Keywords: Sweet taste / Monatin / Natural compounds / Stereoselective synthesis / Chemoenzymatic synthesis

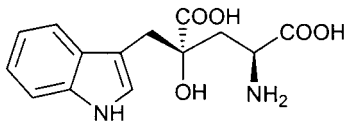
The sweet natural compound monatin **1** has two stereogenic centres and the (2*S*,4*S*) absolute configuration has been attributed to the natural isomer. We obtained all four stereoisomers as pure compounds by a six-step synthetic sequence. The stereogenic centre at C-4 was introduced stereoselectively by a regio- and enantiospecific enzymatic hydrolysis of the racemic ethyl dicarboxylate **4** using a protease from *Aspergillus oryzae*. The absolute configuration of the intermediate products was assigned by X-ray diffraction of chiral derivatives. The stereogenic centre at C-2 was introduced non-specifically, and the resulting diastereomeric mixtures were separated by RP-HPLC. The absolute configurations of the final products were established by comparing retention times on a chiral HPLC column with those of known samples. The four stereoisomers were submitted to tasting trials and three of them, particularly the (2*R*,4*R*) isomer, were found to be intensely sweet. A sample of natural monatin analysed under the same conditions is shown to contain all the four stereoisomers. The relative stereoisomeric content in the plant, as well as the possible isomerisation of the chiral centres during extraction and manipulation of monatin samples, are important points that need to be clarified by extensive analysis of the natural extracts.

(© Wiley-VCH Verlag GmbH & Co. KGaA, 69451 Weinheim, Germany, 2005)

Introduction

Monatin (**1**) is a high-intensity, sweet natural compound isolated from the roots of *Schlerochiton ilicifolius*, a spiny-leaved hardwood shrub growing in the rocky hills of North Western Transvaal (South Africa). Its structure has been elucidated as 4-hydroxy-4-(indol-3-ylmethyl)glutamic acid by Ackerman and co-workers.[1] The same authors also assigned the (2*S*,4*S*) absolute configuration to the natural levorotatory compound based on NOE NMR experiments on a cyclic derivative and the application of the empirical Clough–Lutz–Jirgenson rule, but the crystal structure of the compound is not yet available. The relative sweetness of a mixture of monatin salts, with a prevalence of sodium salt, was estimated to be 1200- or 1400-times that of 5 and 10% sucrose solutions, respectively.

Some synthetic approaches to racemic monatin have been described in the open or patent literature.[2,3] Moreover, some stereoselective syntheses of (–)-monatin have also been reported.[4–6] Since 2002, many patents have been filed concerning monatin synthesis and its possible applications as a sweetener.[7] In one of these patents, monatin has been obtained from a cross aldol reaction of a specific pyruvic acid with oxalacetic or pyruvic acid followed, if necessary, by decarboxylation. Subsequently, the carbonyl group of the ketoglutaric acid compound is then replaced with the amino group.[8] In 2004 the same company obtained monatin by resolution of diastereomeric mixtures, prepared with a modification of the method of Nakamura and coworkers. In another patent the sensory properties of all the four monatin stereoisomers are described.[9] Monatin has also been prepared by complex microbiological processes using mutated D-aminotransferases from glucose, tryptophan and indol-3-lactic acid[10] or 4-(indol-3-ylmethyl)-4-hydroxy-2-oxoglutaric acid.[11]



(2*S*,4*S*)-monatin (**1**)

Monatin is a very interesting compound. It has a modified amino acid structure related to simple chiral synthons

[a] Dipartimento di Scienze Molecolari Agroalimentari – DISMA, Sezione di Chimica, Università degli Studi di Milano, Via Celoria 2, 20133 Milano, Italy
Fax: +39-0250-316801
E-mail: angela.bassoli@unimi.it
[b] School of Chemistry, University of Reading, Whiteknights, Reading RG6 6AD, U.K.

 DOI: 10.1002/ejoc.200400589

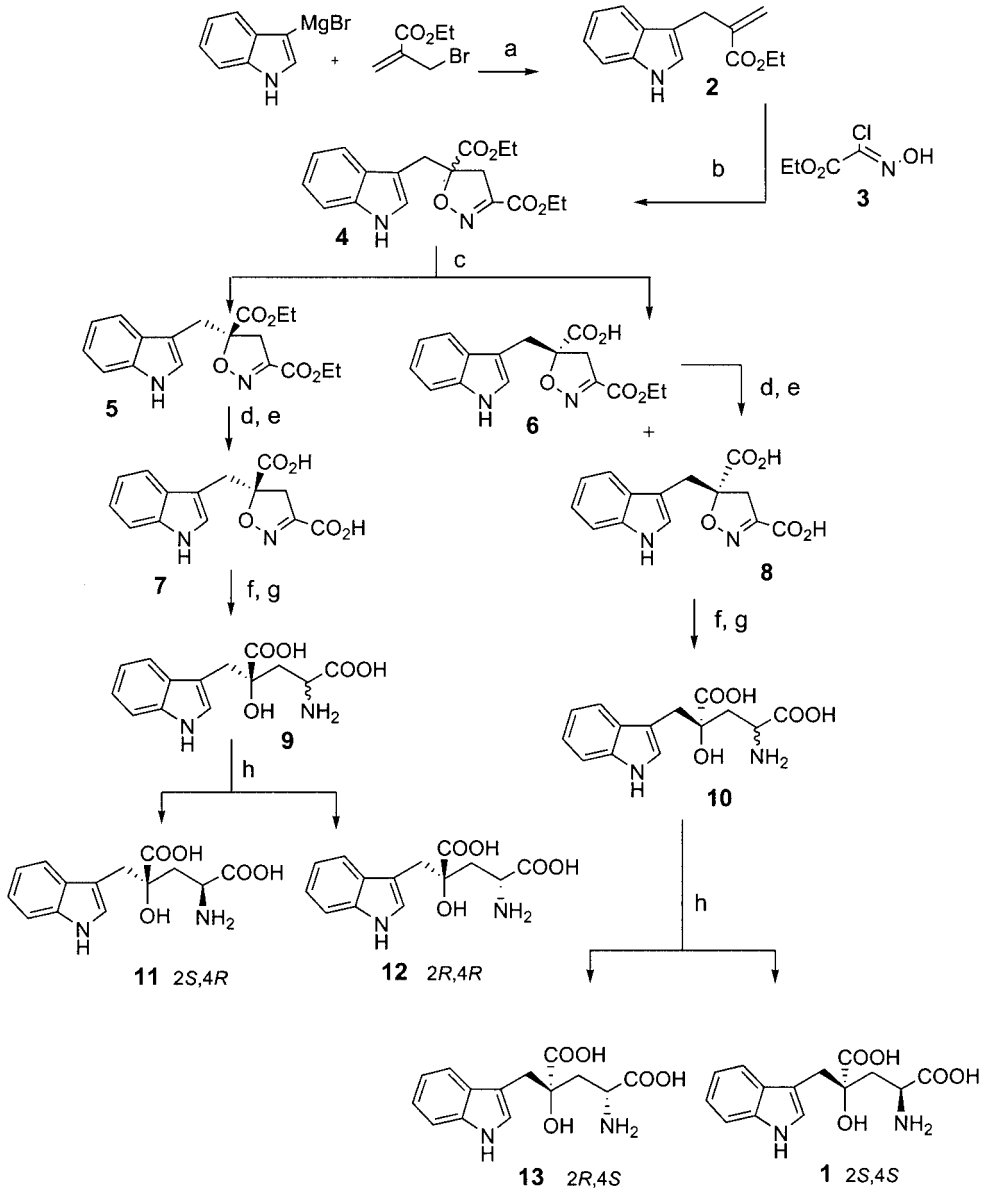
such as glutamic acid and tryptophan, which are themselves taste-active compounds. It is intensely sweet (about three times sweeter than saccharin and six times sweeter than aspartame) and its taste profile is described as extremely pleasant and similar to that of sucrose, making this molecule a good candidate for practical applications as a sugar substitute. Unfortunately, since its discovery only scant information has been published in the open literature due to the difficulties encountered in the synthesis, particularly in the control of the stereochemistry, to the extreme difficulties in obtaining the natural product, and also to the fact that there is a patent pending since 2000 on the utilisation of this compound that discouraged many researchers from further studies.

In this work we describe a new synthetic method to obtain all the four stereoisomers of monatin and the study of their taste activity.

Results and Discussion

The chemoenzymatic synthesis of the four monatin stereoisomers is shown in Scheme 1.

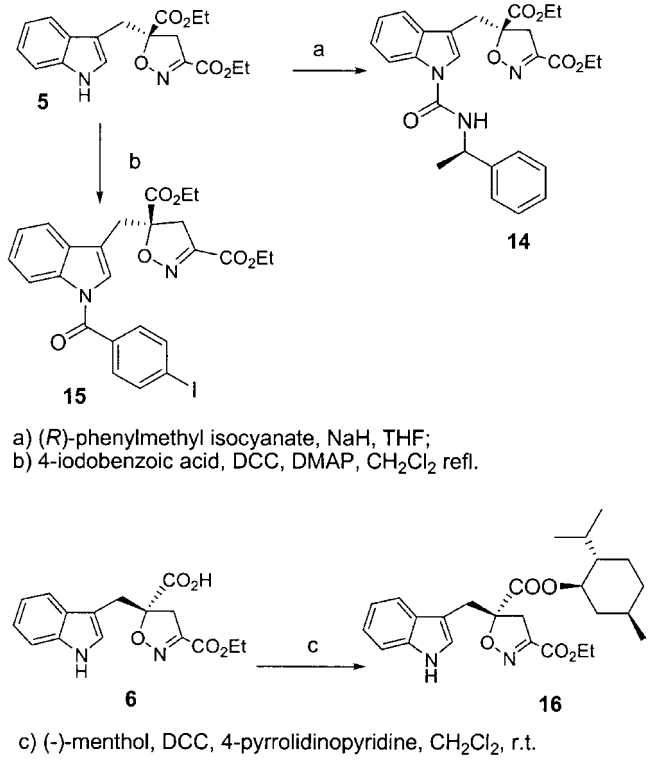
The racemic isoxazoline diester **4** was obtained[2] from the 1,3-dipolar cycloaddition of **2** with the nitrile oxide generated in situ from the chlorooxime **3**. The introduction of the first stereogenic centre was achieved by a chemoenzymatic step. It is known from the literature that proteases from



Scheme 1. Chemoenzymatic synthesis of the four monatin stereoisomers. a) THF, room temp.; b) NEt_3, $CHCl_3$, room temp.; c) proteases from *Aspergillus oryzae*, toluene buffer pH 7; d) KOH/EtOH refl.; e) H^+; f) Na/Hg, EtOH; g) H^+; h) semi-preparative RP18-HPLC resolution.

 © 2005 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

Aspergyllus oryzae are useful in the resolution of sterically demanding esters.[12] We therefore used this enzyme to convert the diester **4** into its monoacid derivative **6** with high regio- and stereoselectivity in an aqueous-organic phase. The obtained monoacid **6** and the unreacted diester **5** were easily separated by extraction. The diester **5** had an *ee* greater than 97%, as shown by direct analysis of the reaction mixture by chiral HPLC (Chiralcel OD). The optical purity of **6** was assessed indirectly by analyzing the corresponding diacid derivative **8** as the corresponding dimethyl ester (see above). The absolute stereochemistries of these intermediates were established by means of derivatisation with appropriate chiral auxiliaries. The diester **5** was treated with (*R*)-1-phenylethyl isocyanate to give the corresponding ureic derivative **14** and with 4-iodobenzoic acid in DCC to give the amide **15** (Scheme 2).



Scheme 2. Synthesis of derivatives **14**, **15** and **16**. a) (*R*)-phenylmethyl isocyanate, NaH, THF; b) 4-iodobenzoic acid, DCC, DMAP, CH_2Cl_2 refl.; c) (–)-menthol, DCC, 4-pyrrolidinopyridine, CH_2Cl_2, room temp.

Both derivatives **14** and **15** crystallise, but only compound **15** gave crystals suitable for X-ray analysis. In this derivative, the introduction of the heavy iodine atom made it possible to resolve the structure and to attribute the (*R*) absolute configuration to the stereogenic centre. This configuration is also that expected on the basis of similar enzymatic resolutions of diesters.

The monoacid **6**, with the opposite configuration, was derivatised with (–)-menthol as a chiral auxiliary and the resulting menthyl ester **16** was also crystallised for X-ray analysis; this showed that the absolute configuration at the stereogenic carbon of the isoxazoline ring is (*S*). Figure 1 shows the crystal structures of derivatives **15** and **16**.

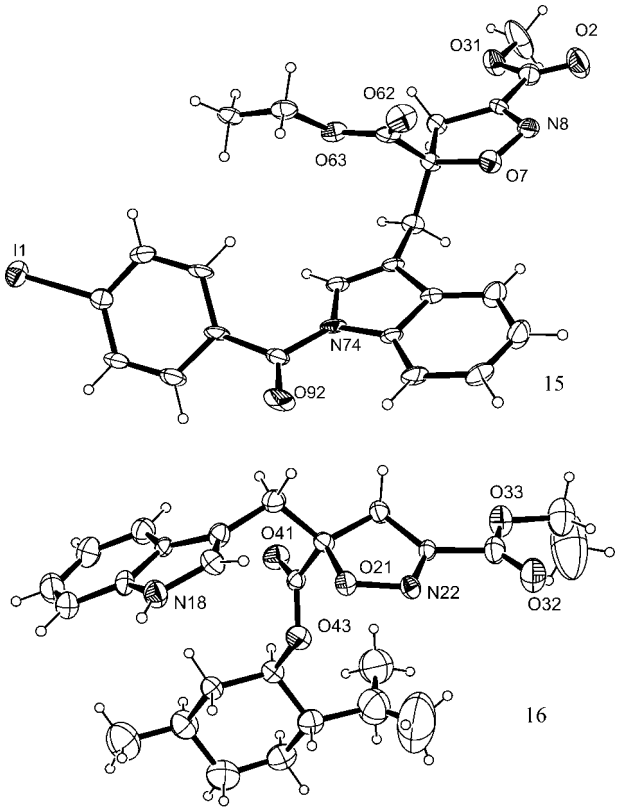


Figure 1. X-ray structures of derivatives **15** and **16** with ellipsoids at 25% probability.

During the enzymatic resolution, the diacid **8** was obtained in variable small amounts depending on the duration of the hydrolysis. This product also has high optical purity (*ee* > 90%), as confirmed by analysis of **8** as the dimethyl ester by chiral HPLC after derivatisation with diazomethane. It is likely that compound **8** is formed from the chemical hydrolysis of (*S*)-**6** after the enzymatic step; in fact, **8** has the same absolute configuration as the monoacid (*S*)-**6**, as shown by the analysis of the CD spectra of pure compounds (*S*)-**6** and **8**, which are very similar and have the opposite sign with respect to that of compound (*R*)-**5** (Figure 2).

The diacid (*S*)-**8** and its enantiomer (*R*)-**7** were then quantitatively obtained by basic hydrolysis of (*S*)-**6** and (*R*)-**5** respectively. Each enantiomer was submitted to hydrogenolysis with Na/Hg in aqueous ethanol; during this step, the second asymmetric centre of the monatin structure was introduced non-specifically and two mixtures of diastereoisomers, **9** and **10**, were obtained. Each mixture was analysed by RP-HPLC and showed the presence of two peaks (P1 and P2 in Figure 3) with identical masses in ESI-LCMS corresponding to an ion at *m*/*z* = 293.2, which is consistent with the structure of monatin. Despite many attempts, we were not able to separate the mixture by column chromatography due to the very similar chromatographic characteristics of the diastereoisomers. Therefore, we used semi-

 © 2005 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

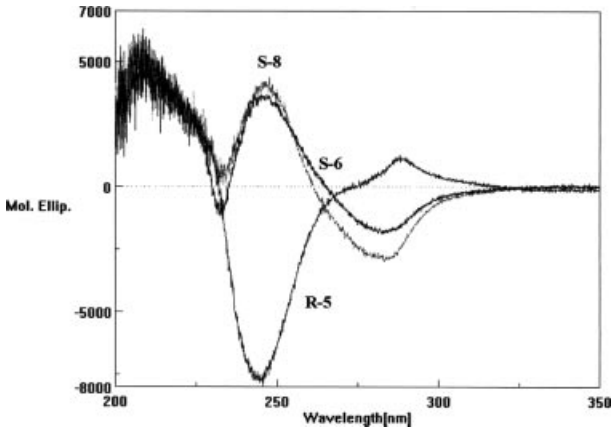


Figure 2. CD spectra of compounds **5**, **6** and **8**.

preparative RP-HPLC to obtain the pure samples after many injections of the mixture and recovery of the separated peaks by lyophilisation of the aqueous organic phase. With this method, 5–15 mg of pure compounds **11** and **12** were obtained from mixture **9**, emanating from (*R*)-**5**; the other diastereoisomers **13** and **1** were obtained from mixture **10**, emanating from (*S*)-**6**.

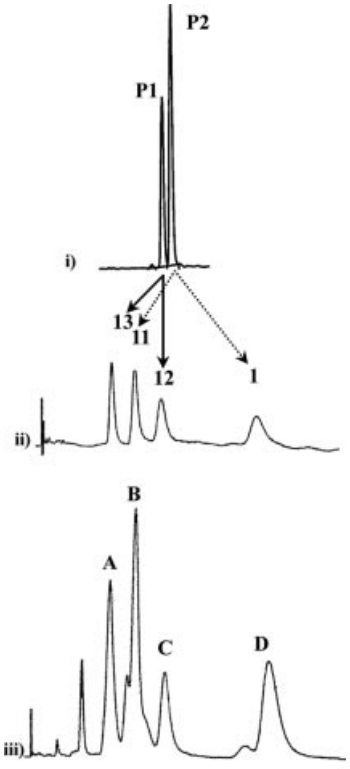


Figure 3. HPLC analysis of monatin stereoisomers; i) reverse-phase analysis of mixture **9** (**10** is identical); ii) Crownpack CR (+), monatin stereoisomers; iii) Crownpack CR (+), natural monatin (same conditions as in ii).

Compounds **1**, **11**, **12** and **13** were submitted to spectral analysis and identified as the four stereoisomers of monatin by means of their spectroscopic characteristics.

While the HRMS and NMR spectroscopic data were fully consistent with those published in the literature,[1] some differences were observed in the optical rotation data. Actually, the reported $[\alpha]_D^{25}$ values are usually referred to monatin sodium or ammonium salt (or their mixture) and using water, aqueous 1 N HCl or 5% NH_3 as a solvent. We isolated monatin and its stereoisomers as free amino acids, and we observed a certain instability of these compounds in acidic conditions due to the formation of lactones and/or lactams, as evidenced by HPLC and LCMS traces. For these reasons we measured $[\alpha]_D^{25}$ in methanol, in which the free amino acids (but not the sodium salts) are soluble.

Owing to the different conditions used, a direct comparison of these data with those published in the literature is difficult; for this reason the assignments of absolute configuration in our samples were not based on optical rotation data, but were obtained by means of chiral HPLC.

In order to confirm the attribution of the absolute configurations to the four samples, X-ray data or a direct comparison with a specimen of natural (2*S*,4*S*)-monatin were necessary. Many attempts to crystallise derivatives **1** and **11**–**13** failed, so we looked for a sample of natural monatin. Natural monatin is not available commercially; the plant *Schlerochiton ilicifolius* grows only in a restricted area in South Africa and is not available from any commercial sources. After many enquiries with companies and scientists who have previously worked on monatin, we were finally able to obtain a few milligrams of an extraction sample. When this sample was first analysed by RP-HPLC we were surprised to find that it consisted of two peaks, chromatographically identical to our P1 and P2, thus indicating the presence of at least two diastereoisomers. Then, the same sample was analysed by chiral HPLC using a method developed by Ajinomoto researchers to resolve the four stereoisomers with high efficiency and previously used to attribute the absolute configuration to the same four stereoisomers synthesised by an independent method.[9] Our compounds were also analysed under the same conditions. The results of this comparison are shown in Figure 3 and in Table 1.

From these results we could attribute the (2*S*,4*S*) configuration to our compound **1**, corresponding to peak D (Figure 3) of the natural monatin sample. Its enantiomer **12** has the (2*R*,4*R*) configuration, while **11** is (2*S*,4*R*) and **13** is (2*R*,4*S*).

It is remarkable to note that the sample of natural monatin we analysed consisted of a mixture of the four stereoisomers, with a predominance of the (2*R*,4*R*) enantiomer. This was unexpected, since natural monatin was reported to be the (2*S*,4*S*) enantiomer.[1] Owing to the small amounts available and to the impossibility of obtaining a larger sample or information about the exact origin and detailed extraction methodology, it is not possible to establish whether these isomers are indeed already present in the plant or come from some racemisation during the processing of the sample. This point should undoubtedly be investigated further in the future.

 © 2005 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

Table 1. HPLC retention times [min] of the compounds obtained in this paper, natural monatin and Ajinomoto's samples of the four stereoisomers. The approximate peak ratio was calculated from the percentage area of the single peaks.

	Compounds in this paper				Natural sample				Ajinomoto's chiral standards[9]			
	13	**11**	**12**	**1**	P1		P2		2*R*,4*S*	2*S*,4*R*	2*R*,4*R*	2*S*,4*S*
Dynamax RP 18	9.29	9.29	10.84	10.84	9.30		10.86					
peak ratio					1.0		2.0					
Inertsil ODS80	–	9.66	12.26	12.26	9.64		12.06		9.64	9.64	12.06	12.06
peak ratio					1.0		2.3					
					A	B	C	D				
Crownpack CR-(+)	12.83	20.82	16.00	35.68	12.32	20.28	15.91	35.46	12.46	20.56	16.24	36.20
peak ratio					1.67	1.0	2.5	2.0				
area%					(20)	(12)	(30)	(24)				

Table 2. Experimental sensory analysis data for the four stereoisomers of monatin.

Sensory analysis data[a]	**13** (2*R*,4*S*)	**11** (2*S*,4*R*)	**12** (2*R*,4*R*)	**1** (2*S*,4*S*)	Ref.
RS (10^{-4} M in H_2O)	250 (1.3)	tasteless (1.3)	1000 (3.5)	350 (2.6)	this paper
RS*	1300	300	2700	50	[8]
RS				1200	[1]
RS		slightly sweet (?)		sweet	[4]

[a] RS = Relative sweetness. * = Relative sweetness values of monatin Na salts compared to the 5% sucrose solution. The optical purity of these stereoisomers is more than 99%.

Pure compounds **1**, **11**, **12** and **13** were then submitted to tasting trials. We used the "sip and spit" procedure[13] with an untrained and informed panel of 4–6 people using water solutions of known concentration and sucrose 3% solution as a standard to determine the relative sweetness (RS) of the compounds. The results of sensory analysis are shown in Table 2.

In our tasting trials three of the four stereoisomers were found to be cleanly sweet, with a small delay time, fast disappearance of the sweet taste and no relevant aftertaste. Compound **11** was found to be tasteless at the examined concentration. This stereoisomer has been described previously as "slightly sweet" by Goodman,[4] but contamination with natural monatin was suspected by the authors; recently, this isomer has also been tasted and described as "slightly sweet with bitter" but no quantitative information on the concentrations used in the tasting trials is provided.[10] Interestingly, the most potent isomer was not the (2*S*,4*S*) isomer but its (2*R*,4*R*) enantiomer, compound **12**. The sweet taste of (2*R*,4*R*)-monatin, as well as the higher potency with respect to its enantiomer, have also been described in a patent by Ajinomoto[11] filed during the preparation of this manuscript. The fact that three (or four) stereoisomers of the same molecule are sweet is not so common, since usually a high stereospecificity of the interaction with the receptor is observed. This fact is very important for practical applications, since synthetic access to single stereoisomers is much more difficult than to racemates. For the same reason, the identification of all the four stereoisomers in a sample of natural monatin and the prevalence of the (2*R*,4*R*) isomer, which is also the sweetest of the series, is very important.

The relative stereoisomeric content in the plant, as well as the possible isomerisation of the chiral centres during extraction and manipulation of monatin samples, are important points that need to be clarified by extensive analysis of the natural extracts as soon as they become available.

The results of the sensory evaluation can also be investigated from the theoretical point of view. In fact, it is possible to explain the observed stereoselectivity by using some of the available sweet-taste receptor models to study the interactions between monatin and its stereoisomers and the putative receptor at molecular level. This has in fact been the object of further studies by our research group, which will be reported shortly.

Experimental Section

General: Reagents were of commercial grade purity; solvents were dried with standard procedures; melting points are uncorrected. ^{1}H NMR spectra were recorded on Bruker AMX-300 and AMX-600 instruments, using TMS as internal standard; *J* values are given in hertz. Mass spectra were recorded on a Finnigan 4021 spectrometer; HPLC experiments were performed with a Varian PROSTAR instrument; HPLC-MS analysis was performed with an Agilent SL 1100 series instrument with ESI and ion-trap system. HRMS were recorded with a Bruker Daltonics APEX II ICR-FTMS instrument, using the ESI ionisation mode. CD spectra were recorded with a Jasco J 810 spectropolarimeter. The crystal structures of **15** and **16** were determined from diffraction data acquired with a Marresearch Image Plate system and refined by full-matrix least-squares to give *wR*1 values of 0.0664, 0.0884 respectively.
CCDC-252171 (for **15**) and -252172 (for **16**) contain the supplementary crystallographic data for this paper. These data can be

 © 2005 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

Synthesis of Monatin Stereoisomers: The synthesis of compounds **2**,[2] **3**[14] and **4**[2] (Scheme 1) has been described in the literature; the hydrogenolysis reactions of diacid isoxazolines **9** and **10** have been reported[2] on the racemic mixture.

Enzymatic Hydrolysis of Diethyl 5-(3-Indolylmethyl)-4,5-dihydroisoxazole-3,5-dicarboxylate (4): Protease (250 mg) from *Aspergillus oryzae* (Sigma type XXII, 3.6 U/mg) was suspended in a two-phase system prepared from phosphate buffer (25 mL, 0.1 M, pH 7.0) and toluene (19 mL). Substrate **4** (1.09 g, 3.2 mmol) in toluene (6 mL) was added and the pH was kept constant by addition of 0.1 N NaOH. The reaction was monitored by chiral HPLC analysis of the organic phase. After 80 h the phases were separated, the organic phase was dried with Na_2SO_4 and the solvent removed in vacuo to obtain 453 mg of **5** (41%). The aqueous phase was acidified to pH 3 with 5% HCl and extracted with AcOEt. The organic phase was washed with brine, dried with Na_2SO_4, filtered and the solvent removed under reduced pressure. The crude product was purified by flash chromatography (eluent: CH_2Cl_2/MeOH, 7:3) to obtain 242 mg of **6** (24%).

Diethyl (3*R*)-5-(3-indolylmethyl)-4,5-dihydroisoxazole-3,5-dicarboxylate (5): M.p. 104–105 °C. $[\alpha]_D^{25}$ = –55.1 (*c* = 0.59, $CHCl_3$); *ee* > 97% (HPLC, Chiralcel OD). ^{1}H NMR ($CDCl_3$): δ = 1.3 (2t, *J* = 7.2 Hz, 6 H, OCH_2CH_3), 3.38 (dd, *J* = 18.7 Hz, 2 H, CH_2), 3.47 (dd, *J* = 15, *J* = 21.4 Hz, 2 H, indole-CH_2), 4.25 (2q, *J* = 7.2 Hz, 4 H, OCH_2CH_3), 7.1–7.7 (m, 5 H, indole), 8.1 (br. s, 1 H, NH) ppm. MS: *m*/*z* (%) = 344 (20) [M^+], 299 (10), 271 (15), 243 (40), 130 (100).

Ethyl (3*S*)-5-(3-Indolylmethyl)-5-carboxy-4,5-dihydroisoxazole-3-carboxylate (6): M.p. 91–92 °C. $[\alpha]_D^{25}$ = +3.75 (*c* = 0.24, MeOH); *ee* > 87%. ^{1}H NMR ([D$_6$]acetone); δ = 1.25 (t, *J* = 7.3 Hz, 3 H, OCH_2CH_3), 2.85 (m, 2 H, indole-CH_2), 3.3 (m, 2 H, CH_2), 4.25 (q, *J* = 7.3 Hz, 2 H, OCH_2CH_3), 6.9–7.7 (m, 5 H, indole) ppm. MS: *m*/*z* (%) = 316 (M^+, 20), 272 (40), 255 (18), 203 (15), 181 (15), 167 (15), 130 (100), 103 (5), 77 (5).

(3*R*)-5-(3-Indolylmethyl)-4,5-dihydroisoxazole-3,5-dicarboxylic Acid (7): A solution of KOH 10% (3.54 mmol) was added to a solution of **5** (405 mg, 1.56 mmol) in 9 mL of aqueous 80% EtOH. After 2.5 h at room temperature and in the dark the mixture was acidified to pH 3 by addition of 10% HCl, treated with brine and extracted with AcOEt. The organic phase was dried with Na_2SO_4, filtered and the solvent evaporated in vacuo to give 208 mg of diacid **7** (62%), m.p. 166–168 °C. $[\alpha]_D^{25}$ = –6.7 (*c* = 0.18, MeOH). ^{1}H NMR ($CDCl_3$, [D$_6$]acetone): δ = 3.4 (dd, *J* = 18.7 Hz, 2 H, CH_2), 3.45 (s, 2 H, indole-CH_2), 6.8–7.5 (m, 5 H, indole), 10.15 (br. s, 1 H, NH) ppm. MS: *m*/*z* (%) = 244 (18), 227 (20), 199 (10), 154 (37), 131 (20), 130 (100), 103 (10), 89 (10), 77 (18), 63 (10), 51 (10).

(3*S*)-5-(3-Indolylmethyl)-4,5-dihydroisoxazole-3,5-dicarboxylic Acid (8): A solution of 10% KOH (1.77 mmol) was added to a solution of **6** (270 mg, 0.78 mmol) in 6 mL of aqueous 80% EtOH. After 2.5 h at room temperature in the dark the mixture was acidified to pH 3 by addition of 10% HCl, treated with brine and extracted with AcOEt. The organic phase was dried with Na_2SO_4, filtered and the solvent evaporated in vacuo to give 139 mg of diacid **8** (62%), m.p. 166–168 °C. $[\alpha]_D^{25}$ = +4.3 (*c* = 0.11, MeOH). ^{1}H NMR ($CDCl_3$ with [D$_6$]acetone): δ = 3.4 (dd, *J* = 18.7 Hz, 2 H, CH_2), 3.45 (s, 2 H, indol-CH_2), 6.8–7.5 (m, 5 H, indole), 10.15 (br. s, 1 H, NH) ppm. MS: *m*/*z* (%) = 244 (18), 227 (20), 199 (10), 154 (37), 131 (20), 130 (100), 103 (10), 89 (10), 77 (18), 63 (10), 51 (10).

Diethyl 5-[1-(1-Phenylethylcarbamoyl)-2,3-dihydro-1*H*-indol-3-ylmethyl]-4,5-dihydroisoxazole-3,5-dicarboxylate (14): (*R*)-(+)-1-Phenylethyl isocyanate (102 mg, 0.69 mmol) was added to a solution of **5** (200 mg, 0.58 mmol) and 60% NaH (24 mg, 0.58 mmol) in dry THF cooled to 0 °C. The reaction was warmed up to room temperature and, after 2 h, heated at reflux for 4 h. A solution of brine was added, the THF evaporated in vacuo and the aqueous phase extracted with AcOEt, dried with Na_2SO_4, and the solvent evaporated. The crude product was purified by flash chromatography (hexane/AcOEt, 7:3) and crystallised from cyclohexane/CH_2Cl_2 to give 240 mg of pure diester **14** (84%). M.p. 78–80 °C. $[\alpha]_D^{25}$ = –7.5 (*c* = 0.64, MeOH). ^{1}H NMR ($CDCl_3$): δ = 1.21 (t, *J* = 7.17 Hz, 3 H, OCH_2CH_3), 1.35 (t, *J* = 7.17 Hz, 3 H, OCH_2CH_3), 1.62 (d, *J* = 6.80 Hz, 3 H, CH_3), 3.21 (d, *J* = 18.2 Hz, 1 H), 3.42 (2d, *J* = 15.26 Hz, 2 H), 3.61 (d, *J* = 18.2 Hz, 1 H), 4.18 (q, *J* = 7.17 Hz, 2 H, OCH_2CH_3), 4.28 (q, *J* = 7.17 Hz, 3 H, OCH_2CH_3), 5.22 (m, 1 H, CH), 5.82 (d, *J* = 6.99 Hz, 1 H, NH), 7.10–7.50 (m, 8 H, indole and Ar), 7.52 (d, *J* = 7.54 Hz, 1 H, Ar), 8.05 (d, *J* = 8.27 Hz, 1 H, Ar) ppm. MS: *m*/*z* (%) = 491 (30) [M – 2], 473 (5), 403 (5), 388 (30), 345 (10), 146 (5), 145 (40), 105 (40), 91 (100), 77 (60).

Diethyl 5-[1–4-Iodobenzoyl)-2,3-dihydro-1*H*-indol-3-ylmethyl]-4,5-dihydroisoxazole-3,5-dicarboxylate (15): DCC (400 mg, 1.94 mmol) and a catalytic amount of DMAP were added to a solution of 4-iodobenzoic acid (471 mg, 1.9 mmol) in dry CH_2Cl_2 (5 mL) under nitrogen. After 1 h at reflux a solution of **5** (98 mg, 0.286 mmol) in dry CH_2Cl_2 (2.3 mL) was added, the reaction mixture heated at reflux for three days. It was then filtered and the solvent evaporated in vacuo. The crude product was purified by flash chromatography (hexane/AcOEt, 8:2) to give 98.5 mg of **15** as a white solid (60%). M.p. (EtOH) 111–114 °C. $[\alpha]_D^{25}$ = –11.0 (*c* = 0.79, CH_2Cl_2). ^{1}H NMR ($CDCl_3$): δ = 1.25 (t, *J* = 7.2 Hz, 3 H, CH_3), 1.35 (t, *J* = 7.2 Hz, 3 H, CH_3), 3.18 (d, *J* = 18.5 Hz, 1 H), 3.48 (2d, *J* = 15.6 Hz, 2 H, indole-CH_2), 3.58 (d, *J* = 18.5 Hz, 1 H), 4.18 (q, *J* = 6.7 Hz, 2 H, CH_2) 4.28 (q, *J* = 6.7 Hz, 2 H, CH_2), 7.10–8.50 (m, 9 H, indole and ar) ppm. MS: *m*/*z* (%) = 574 (5) [M^+], 360 (40), 231 (100), 203 (20), 104 (15), 76 (18).

3-Ethyl 5-(2-Isopropyl-5-methylcyclohexyl)-5-(2,3-dihydro-1*H*-indol-3-ylmethyl)-4,5-dihydroisoxazole-3,5-dicarboxylate (16): DCC (55 mg, 0.27 mmol), a catalytic amount of DMAP and (–)-menthol (46 mg, 0.29 mmol) were added to a stirred solution of **6** (85 mg, 0.27 mmol) in 5.7 mL of CH_2Cl_2. The reaction mixture was stirred at room temperature for 3 d. The organic phase was washed with water and dried with Na_2SO_4, filtered and the solvents evaporated. The crude product was purified by flash chromatography (hexane/AcOEt, 7:3) to give 63 mg of **16** (52%). M.p. (hexane/CH_2Cl_2) 118–119 °C. $[\alpha]_D^{25}$ = –2.4 (*c* = 1.45, CH_2Cl_2). ^{1}H NMR ($CDCl_3$): δ = 0.67 (d, *J* = 6.75 Hz, 3 H, CH_3), (d, *J* = 6.75 Hz, 3 H, CH_3), 0.90 (d, *J* = 6 Hz, 3 H, CH_3), 1.28 (t, *J* = 6.7 Hz, 3 H), 1.20–1.90 (m, 9 H, menthyl), 3.18 (dd, *J* = 18.7 Hz, 1 H), 3.48 (2d, *J* = 15 Hz, 2 H, CH_2-indole), 3.58 (d, *J* = 18.7 Hz, 1 H), 4.28 (q, *J* = 6.7 Hz, 2 H), 4.73 (m, 1 H), 7.10–7.65 (m, 5 H, indole), 8.1 (br. s, 1 H, NH) ppm. MS: *m*/*z* (%) = 454 (40) [M^+], 317 (5), 271 (5), 243 (20), 197 (5), 130 (100), 83 (5).

The hydrogenolysis of isoxazoline diacids **7** and **8** gave a mixture of diastereoisomers **9** (71%) and **10** (60%) respectively. The mixtures were resolved by semi-preparative HPLC.

(2*R*,4*R*)-Monatin (12): $[\alpha]_D^{25}$ = –3.85 (*c* = 0.78, MeOH). ^{1}H NMR (D_2O): δ = 2.05 (dd, *J* = 15.2, *J* = 11.7 Hz, 1 H, H-3a), 2.70 (dd, *J* = 15.2, *J* = 2.0 Hz, 1 H, H-3b), 3.08 (d, *J* = 14.7 Hz, 1 H, H-5a), 3.28 (d, *J* = 14.7 Hz, 1 H, H-5b), 3.63 (dd, *J* = 11.7, *J* = 2.0 Hz, 1 H, H-2,), 7.0–7.8 (m, 5 H, indole) ppm. LCMS (ESI): *m*/*z* (%) = 293 (100) [M + 1], 274 (5), 257 (10), 121 (40). HRMS (ESI, pos):

 © 2005 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

m/*z* (%) = 293.11219 [M + 1], 315.09451 [M + Na]; calculated: 293.11320.

(2*S*,4*R*)-Monatin (11): $[\alpha]_D^{25}$ = –0.7 (*c* = 0.43, MeOH). ^{1}H NMR (D_2O): δ = 2.21 (dd, *J* = 15.2, *J* = 9.9 Hz, 1 H, H-3a), 2.44 (dd, *J* = 15.2, *J* = 3.2 Hz, 1 H, H-3b), 3.22 (dd, *J* = 14.4 Hz, 2 H, H-5), 3.92 (dd, *J* = 9.9, *J* = 3.2 Hz, 1 H, H-2), 7.0–7.8 (m, 5 H, indole) ppm. LCMS (ESI): *m*/*z* (%) = 293 (100) [M + 1], 275 (5), 274 (5). HRMS (ESI, pos): *m*/*z* (%) = 293.11212 [M + 1], 315.09491 [M + Na]; calculated: 293.11320.

(2*R*,4*S*)-Monatin (13): $[\alpha]_D^{25}$ = +10.0 (*c* = 0.02 MeOH). ^{1}H NMR (D_2O): δ = 2.21 (dd, *J* = 14.9, *J* = 9 Hz, 1 H, H-3a), 2.44 (dd, *J* = 15.2, *J* = 3.2 Hz, 1 H, H-3b), 3.22 (dd, *J* = 14.4 Hz, 2 H, H-5), 3.92 (dd, *J* = 9.9, *J* = 3.2 Hz, 1 H, H-2), 7.0–7.8 (m, 5 H, indole) ppm. LCMS (ESI): *m*/*z* (%) = 291 (100) [M – 1], 211 (5). HRMS (ESI, pos): *m*/*z* (%) = 293.11212 [M + 1], 315.09491 [M + Na]; calculated: 293.11320.

(2*S*,4*S*)-Monatin (1): $[\alpha]_D^{25}$ = +1.2 (*c* = 0.93, MeOH). ^{1}H NMR (D_2O): δ = 2.05 (dd, *J* = 15.2, *J* = 11.7 Hz, 1 H, H-3a), 2.70 (dd, *J* = 15.2, *J* = 2.0 Hz, 1 H, H-3b), 3.08 (d, *J* = 14.7 Hz, 1 H, H-5a), 3.28 (d, *J* = 14.7 Hz, 1 H, H-5b), 3.63 (dd, *J* = 11.7, *J* = 2.0 Hz, 1 H, H-2), 7.0–7.8 (m, 5 H, indole) ppm. LC-MS (ESI): *m*/*z* (%) = 291 (100) [M – 1], 113 (5). HRMS (ESI, pos): *m*/*z* (%) = 293.11219 [M + 1], 315.09451 [M + Na]; calculated: 293.11320.

HPLC Analysis: All the compounds were analysed by RP-HPLC for their chemical purity. The enantiomeric excess was determined for compounds **5** and **6** with a Chiralcel OD column from Daicel. The four stereoisomers of monatin **1**, **11**, **12** and **13** were analysed by Dr. Yusuke Amino, Ajinomoto Co. Inc., with a Crownpack CR (+) (Daicel) column in comparison with samples obtained by an independent method[8] and with a sample of natural monatin. Peaks were identified based on retention times and enrichment with reference samples. Analytical conditions: Dynamax C18 25×0.4 mm id.; eluent A: H_2O+0.1% acetic acid; eluent B: CH_3CN+0.1% acetic acid; t_0 A:B = 90:10, t_{25min} A:B = 75:25, flow 1.0 mL/min, UV 254 nm. Chiralcel OD, 25×0.4 id., Daicel; eluent: hexane/*i*PrOH (65:35) isocratic conditions, flow 0.8, UV 254 nm. Crownpack CR(+) 15×0.4 id., Daicel; eluent $HClO_4$ aq (pH 2.00)/MeOH (85:15), flow 1.1, UV 210 nm.

Acknowledgments

We thank Dr. Yusuke Amino (Ajinomoto Co. Inc., Japan) for HPLC analysis on chiral columns, Dr. Ulrich ter Meer (Nutrinova Nutrition Specialties and Food Ingredient GmbH, Germany) for helpful discussions, MIUR (Italian Ministry for the Research and University) and University of Milan for financial support (programs COFIN 2002 and FIRST 2003), Fondazione Fratelli Confalonieri (Milano) for a PhD fellowship to G. Busnelli and HFSP for a short fellowship to A. Bassoli. We thank EPSRC (U.K.) and the University of Reading for funds for the Image Plate system.

[1] R. Vleggaar, L. G. J. Ackerman, P. S. Steyn, *J. Chem. Soc., Perkin Trans. 1* **1992**, 3095–3098.
[2] C. H. Holzapfel, K. Bischofberger, J. Olivier, *Synth. Commun.* **1994**, *24*, 3197–3211.
[3] US Pat. 5994559 A, Nov 30, **1999**.
[4] K. Nakamura, T. J. Baker, M. Goodman, *Org. Lett.* **2000**, *2*, 2967–2970.
[5] O. Tamura, T. Shiro, H. Toyo, H. Ishibashi, *Chem. Commun.* **2003**, 2678–2679.
[6] K. Nakamura, K. Kogiso, T. Nakajima, H. Kayahara, *Peptide Sci.* **2004**, *40*, 61–64.
[7] WO2003091396; EP Pat. 1350791; WO2003056026; JP Pat. 2003171365; US Pat. 2002197371; WO2002087358; JP Pat. 2002060382.
[8] WO2003059865.
[9] WO2003045914 (JP); EP 1449832 (ENG).
[10] WO2003091396.
[11] WO2004053125.
[12] S. Yang, W. Hayden, K. Faber, H. Griengl, *Synthesis* **1992**, 365–366.
[13] A. Arnoldi, A. Bassoli, G. Borgonovo, M. G. B. Drew, L. Merlini, G. Morini, *J. Agr. Food Chem.* **1998**, *46*, 4002–4010.
[14] A. P. Kozikowski, M. Adamczy, *J. Org. Chem.* **1983**, *48*, 366–372.

Received: August 18, 2005

 © 2005 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim