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Preparation and Characterization of Four Stereoisomers of Monatin

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Monatin is a naturally occurring, sweet amino acid comprising four stereoisomers due to its two asymmetric centers at C2 and C4. However, the characteristics of each stereoisomer have not yet been fully investigated. To obtain a sufficient amount of racemic monatin for optical resolution, a synthetic method was developed by modifying a possible biosynthetic pathway, *i.e.*, a cross-aldol reaction and subsequent transamination. The key intermediate, 4-hydroxy-4-(3-indolylmethyl)-2-ketoglutaric acid, was obtained *via* the cross-aldol reaction of pyruvic acid and indole-3-pyruvic acid. Subsequently, the carbonyl group was converted to a hydroxyimino group through reaction with hydroxylamine and then to an amino group *via* hydrogenation to produce monatin. Next, the racemic monatin was divided into mixtures of two pairs of enantiomers through recrystallization. Finally, both enantiomers of the *N*-carbobenzoxy-γ-lactone derivatives of monatin were separated by preparative HPLC and deprotected. It was found that all optically pure stereoisomers exhibited a sweet taste. The isomer that displayed the most intense sweetness was the (2*R*,4*R*)-isomer, as determined by single crystal X-ray structure analysis of the monatin potassium salt, whereas the least sweet isomer was the (2*S*,4*S*)-isomer, which demonstrated a far lower sweetness than was previously reported.

Key words monatin; stereoisomer; sweet taste; cross-aldol reaction; natural compound

Monatin is a naturally occurring amino acid derivative isolated from the bark of the roots of *Schlerochiton ilicifolius*, which is a plant native to the north western Transvaal of South Africa. The structure of monatin was reported as (2*S*,4*S*)-2-amino-4-carboxy-4-hydroxy-5-(3-indolyl)-pentanoic acid ((2*S*,4*S*)-4-hydroxy-4-(3-indolylmethyl)-glutamic acid (refer to structural formula (**1a**) in Fig. 1) by Vleggaar *et al.*[1] Discrepancies in the numbering system for monatin have caused some confusion in the case of the (2*S*,4*R*)- and (2*R*,4*S*)-diastereomers. In this report, we assigned the α carbon of glutamic acid as C2. Thus, monatin is numbered in accordance with other substituted amino acid derivatives.

According to Vleggaar *et al.*, the sweetness of (2*S*,4*S*)-monatin (**1a**) (natural-type monatin) derived from the natural plant is reported to be 1200- to 1400-fold more intense than that of sucrose. The (2*S*,4*S*) absolute configuration of natural monatin was assigned by nuclear Overhauser effect (NOE) NMR spectroscopic experiments based on its cyclic derivative and the empirical Clough–Lutz–Jirgenson rule. These results suggested that monatin is an attractive candidate in the search for novel, high-intensity sweeteners.

Various methods have been reported regarding the synthesis of monatin, some of which produce a mixture of stereoisomers,[2–5] while others describe stereoselective syntheses.[6–11] However, there have been no reports wherein all four stereoisomers having the same structural formula as **1a** are synthesized, isolated, and characterized as pure substances. Nakamura *et al.* synthesized and isolated hydrochlorides of **1a** and (2*S*,4*R*)-monatin (**1b**).[6] They reported that, with respect to the intensity of sweetness, synthetic **1a** exhibited a sweetness potency equivalent to that of natural monatin, while synthetic **1b** exhibited only a slightly sweet taste, presumably due to **1a**, which is thought to be present as an impurity; however, the specific intensity of the sweetness was not reported. Kitahara *et al.* reported a selective synthetic method toward each stereoisomer of monatin; however, the degree of sweetness of each stereoisomer was not sufficiently discussed.[7,8] Bassoli *et al.* disclosed the isolation of the four stereoisomers of monatin and sensory analysis of each stereoisomer; however, the chemical and optical purities of each stereoisomer were not sufficiently indicated.[12] In their study, the assignment of stereochemistry of the products was justified by HPLC analysis using a chiral column and carried out by our laboratories.[12]

We have been interested in the taste profiles of other stereoisomers of monatin, particularly that of (2*R*)-isomers, as it is known that (*R*)-amino acids (D-amino acids) such as (*R*)-tryptophan exhibit a sweet taste, while (*S*)-amino acids do not, with the exception of (*S*)-alanine, (*S*)-proline, and (*S*)-serine.[13,14]

As mentioned above, there has been no clear data for the intensity of sweetness of each monatin stereoisomer in practical concentrations corresponding to 5–10% sucrose concentration, with the exception of natural monatin.

Although the development of a mass production method is necessary to thoroughly evaluate monatin stereoisomers, when we began our study, to the best of knowledge there were no reports regarding a large-scale synthetic method of monatin. Homologs of monatin (γ-hydroxy-glutamic acid) in which the 3-indolylmethyl at C4 (γ-position) is replaced by various substituents, including methyl, ethyl, isobutyl, carboxymethyl, or carboxyethyl, are known as natural products.[15–19] These compounds, including monatin, must be produced *via* a com-

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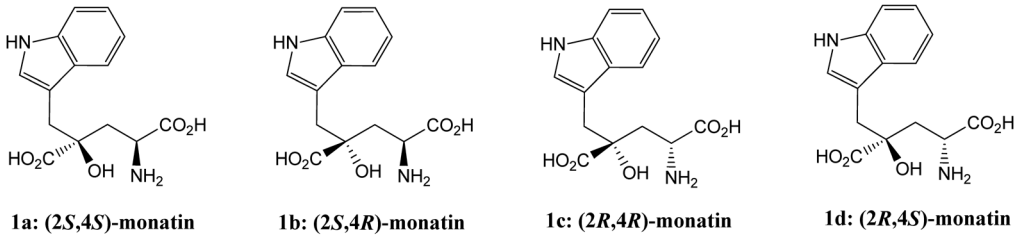


Fig. 1. Structures of the Four Stereoisomers of Monatin

mon biosynthetic pathway; *i.e.*, a cross-aldol reaction and subsequent transamination. The basic structure of monatin is a substituted ketoglutaric acid, which must be constructed by a regiospecific cross-aldol reaction between pyruvic acid and indole-3-pyruvic acid. Early studies on the aldol reaction of pyruvic acid derivatives included the catalytic asymmetric homo-aldol reaction of ethyl pyruvate and cross-aldol reaction of various 2-ketoesters with activated carbonyl compounds in various organic solvents.[20,21] The decarboxylative self-condensation of oxaloacetic acid and self-aldol condensation reaction of pyruvic acid in aqueous solution have also been reported.[22–24] However, to the best of our knowledge, the only example of a cross-aldol reaction of α-keto acids in an aqueous solution is the reaction of glyoxylic acid and oxaloacetic acid.[25,26] In this reaction, glyoxylic acid never underwent self-condensation, allowing for relatively easy production of a single condensed product. In the same report, transformation of the obtained γ-hydroxy α-ketoglutaric acid to γ-hydroxy-L-glutamic acid *via* an aminotransferase was described as an example of the biosynthesis of γ-hydroxy-L-glutamic acid. The development of chemical processes based on this biosynthetic pathway is an attractive approach toward establishing a large-scale synthesis of monatin.

In this paper, we report a novel, practical synthetic method toward racemic monatin, as well as details of the isolation and characterization of each stereoisomer.

Results and Discussion

Preparation of Stereoisomer Standards Mixtures of the (2*S*,4*S*)- and (2*S*,4*R*)-isomers of monatin (**1a**, **b**, respectively) and the (2*R*,4*R*)- and (2*R*,4*S*)-isomers of monatin (**1c**, **d**, respectively) were prepared from (*R*)-Garner's aldehyde and (*S*)-Garner's aldehyde, respectively, according to the method described by Nakamura *et al.*[6] Each stereoisomer (diastereomer) was separated by means of reversed-phase (RP)-HPLC using a C_{18} column. Under these conditions, **1b** and **d** were eluted first, followed by **1a** and **c**, as shown in Fig. 2. While studying the stability of monatin, it was found that strongly acidic conditions were not suitable for characterizing monatin as these conditions induced a structural change to form a mixture of monatin, *i.e.*, the lactone and the lactam; therefore, each isomer of monatin was isolated as the ammonium salt. Using these stereoisomers, we established a chiral HPLC analysis in which the four stereoisomers were clearly resolved into four peaks using a Crownpak CR(+) column. Using this column, each isomer eluted as a single peak when injected separately with the following order of retention times, **1d**>**c**>**b**>**a**, as shown in Fig. 3. Both the optical purities and amounts of obtained stereoisomers using the above procedure were not sufficient to determine the exact sweetness potency of each.

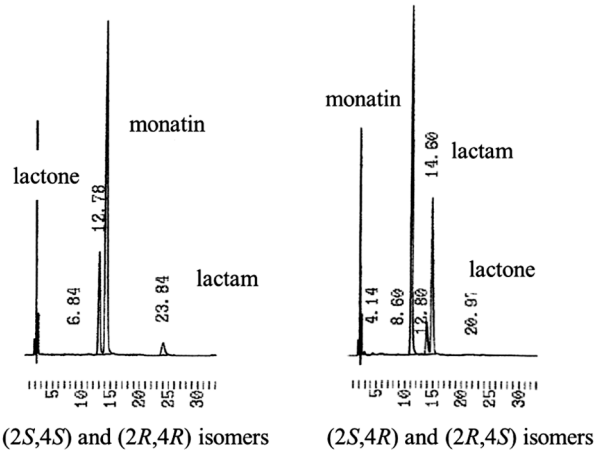


Fig. 2. RP-HPLC Chromatogram of Monatin, Lactone, and Lactam

Left chromatogram: (2*S*,4*S*)- and (2*R*,4*R*)-isomers; eluted in order of lactone, monatin, lactam. Right chromatogram: (2*S*,4*R*)- and (2*R*,4*S*)-isomer; eluted in order of monatin, lactam, and lactone.

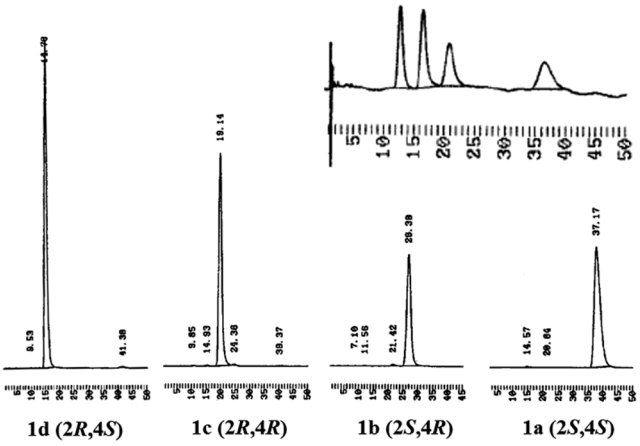


Fig. 3. Resolution of the Four Monatin Stereoisomers *via* Chiral HPLC Using a Crownpak CR(+) Column

Synthesis of Racemic Monatin We carried out a study to develop a chemical synthetic method of monatin through modification of a biosynthetic pathway in an attempt to establish an industrial production method[27] (Chart 1). The first step of our strategy was to construct a basic substituted ketoglutaric acid *via* a regioselective cross-aldol reaction between pyruvic acid (**3**) and indole-3-pyruvic acid (**2**). Subsequently, the carbonyl group of the substituted ketoglutaric acid was replaced by an amino group.

Notably, the reaction of **2** as an acceptor with 5 eq of **3** as a donor generated the desired 4-hydroxy-4-(3-indolylmethyl)-2-ketoglutaric acid intermediate (**5**) in moderate yield under

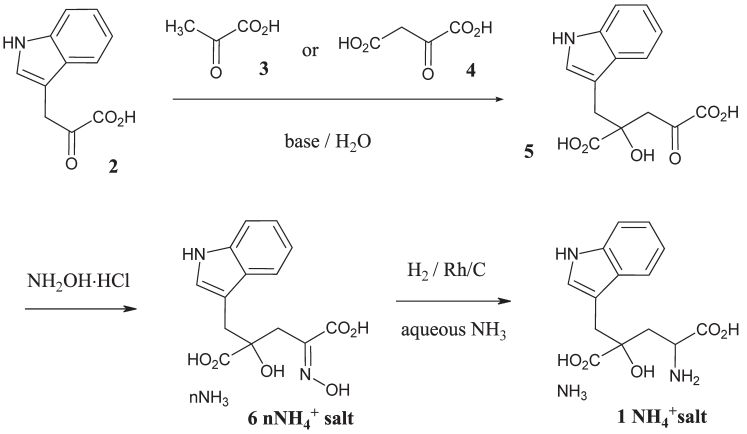
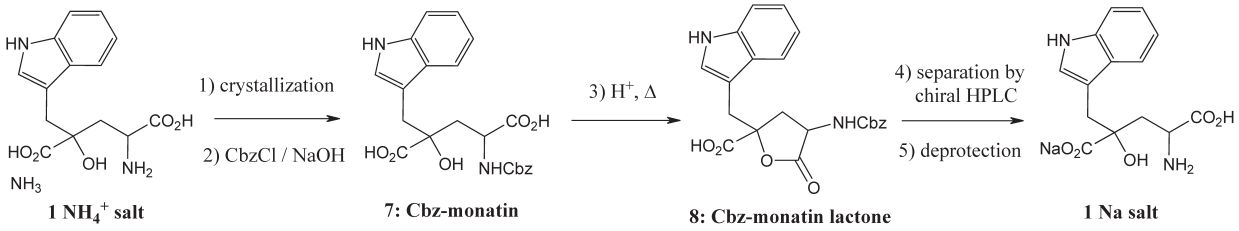


Chart 1. Large-Scale Synthesis of Racemic Monatin



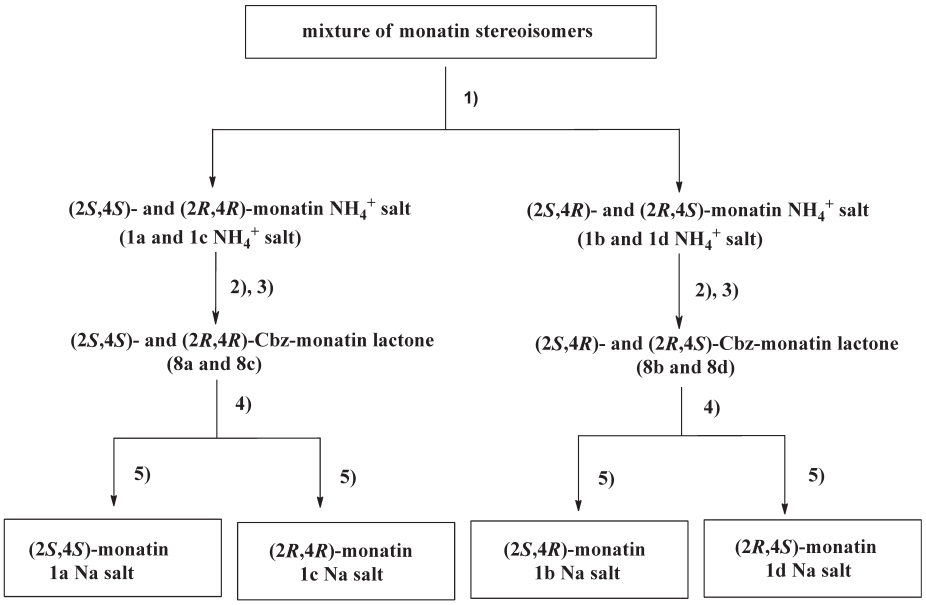


Fig. 4. Separation of Each of the Four Monatin Stereoisomers

basic aqueous conditions; however, there is potential for four condensation products to be generated. Through the use of excess **3**, generation of the self-aldol product of **2** and the product with the reverse arrangement might be suppressed. The decarboxylative cross-condensation of oxaloacetic acid (**4**) and **2** also proceeded in moderate yield. Moreover, **5** was easily transformed to 4-hydroxy-4-(3-indolylmethyl)-2-hydroxyiminoglutaric acid (**6**) upon reaction with hydroxylamine *in situ*, without the need for further purification. The ammonium salt of **6** (**6** NH_4^+ salt) was easily isolated as a single product *via* crystallization from a mixed solvent of aqueous ammonia and 2-propanol. The hydrogenation reaction of **6** with a Rh/C catalyst under a pressure of 1 MPa in aqueous ammonia proceeded smoothly to produce the racemic monatin ammonium salt (**1** NH_4^+ salt) as crystals in good yield. The ratio of the sum of **1a** and **c** to the sum of **1b** and **d** was 60 : 40. Details of this synthesis and application of this strategy to the chiral synthesis of **1c** will be reported in the near future.

Isolation of Each Monatin Stereoisomer We obtained between several tens and one hundred grams of racemic monatin, which we used to determine the optical resolution of each of the four stereoisomers using the following procedure[28] (Fig. 4). Crystals of the major enantiomer mixture of **1a** and **c** were easily obtained in high purity by recrystallization of the

racemic mixture from a mixed solvent of water and ethanol. Conversely, the crystals of another enantiomer mixture of **1b** and **d** were obtained from the mother liquor by recrystallization from the same solvent system. Next, each mixture of enantiomers was converted to the corresponding *N*-carbobenzoxy-monatin (Cbz-monatin, **7**) upon reaction of monatin with carbobenzoxy chloride. Cyclization of **7** was carried out by heating in ethyl acetate at 75°C in the presence of a catalytic amount of *p*-toluenesulfonic acid to give the *N*-carbobenzoxy-*γ*-lactone of monatin (Cbz-lactone, **8**). Each stereoisomer of **8** was then separated by semi-preparative chiral HPLC. Separation of (2*S*,4*S*)- and (2*R*,4*R*)-Cbz-lactone (**8a**, **c**, respectively) was performed using a CHIRALPAK AS column to afford optically pure **8a** and **c**. Separation of (2*S*,4*R*)- and (2*R*,4*S*)-Cbz-lactone (**8b**, **d**, respectively) was performed using a CHIRALCEL OJ column to afford optically pure **8b** and **d**. The four stereoisomers of monatin (**1a–d**) were obtained in excellent purities (>99.2%) as crystals of the sodium salt by hydrogenation with Pd/C, followed by hydrolysis with aqueous sodium hydroxide. Their optical purities were verified by chiral HPLC, the results of which are demonstrated in Fig. 3.

Sweetness of Each Monatin Stereoisomer The four stereoisomers were obtained in excellent purities and submitted to taste testing for the first time. The relative sweetness intensity of the isomers was compared to sucrose, with the results shown in Table 1: **1a**: 50 times (Na salt, compared to 5% sucrose solution), 25 times (K salt, compared to 10% sucrose solution); **1b**: 300 times (Na salt, 5% sucrose), 100 times (Na salt, 10% sucrose); **1c**: 2700 times (Na salt, 5% sucrose), 1700 times (K salt, 10% sucrose); and **1d**: 800 times (Na salt, 5% sucrose), 300 times (Na salt, 10% sucrose). As seen from these results, as is usual with high-potency sweeteners, the relative sweetness changed depending on the concentration of the authentic sucrose solution. Note that the intensity of monatin sweetness was constant regardless of the counter cation. After our study was complete, the sweetness potency of **1c** was reported as approximately 3100 at 5% sucrose equivalent (SE) and 2700 at 8% SE by Fry *et al.*[29] Surprisingly, all isomers were found to exhibit a sweet taste; however, **1a** was found to be the least sweet isomer, while the other three isomers, especially **1c**, were found to be intensely sweet.[28]

When natural monatin was first derived from the plant, it was reported that the intensity of sweetness was 1200- to 1400-fold greater than that of sucrose. Since we have found that the sweetness potency of the (2*S*) form is much weaker than 500-fold, the results suggest that natural monatin should mainly comprise the (2*R*) form to explain its 1200- to 1400-fold sweetness. It is known that a relationship exists between the taste and stereochemistry of amino acids, in which many (*R*)-amino acids (D-amino acids) such as (*R*)-tryptophan exhibit a sweet taste, while (*S*)-amino acids (L-amino acids) are not sweet, with the exception of (*S*)-alanine, (*S*)-proline, and (*S*)-serine.[13,14] This rule can also be broadly applied to **1a** and **c**. It is well-known that the N-terminal *α*-amino group and *β*-carboxyl group of aspartame (L-*α*-aspartyl-L-phenylalanine 2-methyl ester) possess the same alignment as the *α*-amino group and *α*-carboxyl group of sweet D-amino acids.[30] Thus, it is suggested that **1c** and aspartame are likely to interact with a common domain of the sweet taste receptor.

Isomer **1a**, which is expected to be natural monatin and has been reported to exhibit a sweetness in the range of >1000 times that of sucrose, was found to be only 25–50 times as sweet as sucrose. In contrast, **1c** exhibited a sweetness potency of 1700–2700 times that of sucrose. However, these results are difficult to reconcile with the stereochemical assignments made by Vleggaar *et al.* and Bassoli *et al.*, in which they reported that the natural monatin they obtained was a mixture of the four stereoisomers.[12] We do not have any information regarding their detailed extraction methodology; thus, it is not possible to conclude whether these isomers were already present in the plant or if they occurred as a result of racemization during isolation. Taking the sweetness potency of each isomer into account, there is a possibility that the monatin isolated by Vleggaar *et al.* may be a mixture of **1a** and the (2*R*,4*R*)-isomer (**1c**).

The specific rotations for (−)**1a** reported in the literature range from −7.6 to −10.9° (in 1 N HCl). However, we learned from our stability study that monatin is stable under neutral or weakly acidic conditions, but unstable under strongly acidic conditions. In aqueous acid, monatin is able to cyclize to the lactone and subsequently to the lactam, eventually reaching equilibrium (Fig. 5). As the acidity of the solution is increased, the ratio of the lactone form to linear form monatin also increased. Indeed, when a sample of pure monatin was heated at 70°C in pH 3 buffer for an entire day, the RP-HPLC chromatogram showed that the single peak had become heterogeneous, as shown in Fig. 2. The lactone of **1a** and **c** was then injected and shown to elute much sooner than the linear form of monatin. Thus, 1 N HCl (and acidic solutions in general) is not a suitable solvent for characterizing monatin, as these conditions induce a structural change to form mixtures of the monatin lactone and lactam; therefore, we measured the specific rotation of monatin stereoisomers in aqueous ammonia.

Single Crystal X-Ray Structure Analysis of the (2*R*,4*R*)-Monatin (1c) Potassium Salt Dihydrate The **1c** potassium salt was chosen as the subject of this study, due to its relatively high melting point, and was recrystallized from a mixture of ethanol and water by slow evaporation.

The crystal structure of the **1c** potassium salt dihydrate was determined by single crystal X-ray structure analysis. A summary of this crystallographic analysis is shown in Table 2. The asymmetric unit of the crystal was shown to contain one monatin potassium salt and two water molecules. Figure 6 shows the asymmetric unit of the crystal as an ORTEP drawing. The absolute structure of monatin in this crystal was deduced as the (2*R*,4*R*)-form, based on the fact that the Flack parameter[31] converged to a value of 0.082(22) when the refine-

Table 1. Experimental Sensory Analysis Data for the Four Monatin Stereoisomers

Compound	1a	1b	1c	1d
Absolute configuration	(2*S*,4*S*)	(2*S*,4*R*)	(2*R*,4R)	(2*R*,4*S*)
Relative sweetness[1), a)]	1200–1400			
Relative sweetness (5% sucrose)[b)]	50	300	2700	1300
Relative sweetness (10% sucrose)[c)]	25	100	1700	800
Optical purity[d)]	99.8	99.4	99.3	99.2

a) Previously reported relative sweetness value.[1] *b*) Relative sweetness values of monatin sodium salts compared to the 5% sucrose solution. *c*) Relative sweetness values of monatin sodium salts [(2*S*,4*R*) and (2*R*,4*S*)] and potassium salts [(2*S*,4*S*) and (2*R*,4*R*)] compared to a 10% sucrose solution. *d*) Optical purities of the stereoisomers determined by HPLC.

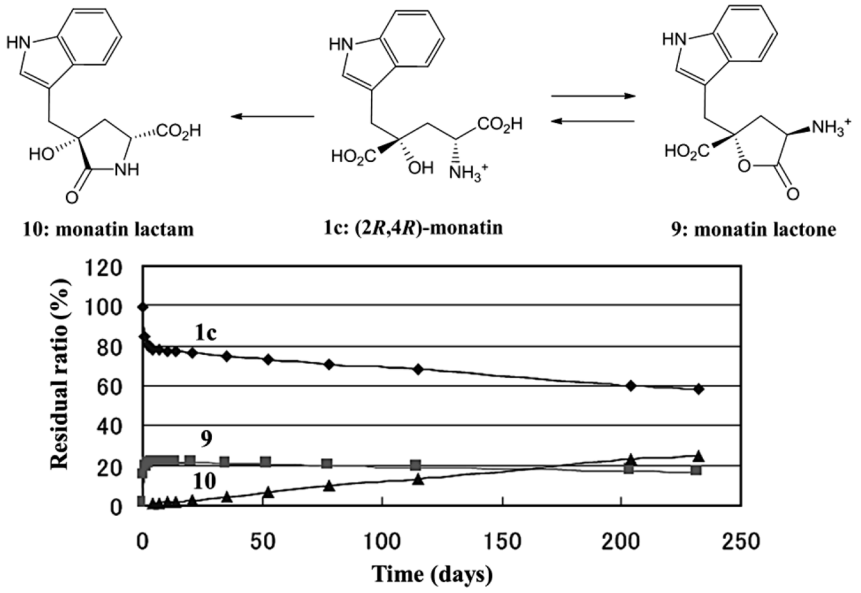


Fig. 5. Intramolecular Reaction of (2*R*,4*R*)-Monatin in Acidic Solution

Stability of (2*R*,4*R*)-monatin (**1c**) in citric acid buffer (0.05 M, pH3.0) at 25°C.

Table 2. General Crystallographic Information for the (2*R*,4*R*)-Monatin Potassium Salt Dihydrate Crystal

Formula unit:		$C_{14}H_{13}N_2O_5K \cdot 2H_2O$
Space group:		$P2_12_12_1$
Unit cell parameters	*a*:	7.762(3) Å
	b:	30.883(3) Å
	c:	6.745(3) Å
Z Value:		4
Calculated density:		1.349 g/cm^3
No. of unique reflections:		1761
No. of parameters refined:		295
Goodness of fit:		1.24
Residuals	$R^{a)}$:	0.051
	$Rw^{b)}$:	0.108
	$R1^{c)}$:	0.036
Max shift error in final cycle:		0.28
Maximum peak in final diff. map:		0.17 e$^-$/Å^3
Minimum peak in final diff. map:		−0.24 e$^-$/Å^3

a) $R=\Sigma\ (F_o^2-F_c^2)/\Sigma\ F_o^2$. *b*) $Rw=(\Sigma\ w(F_o^2-F_c^2)^2/\Sigma\ w(F_o^2)^2)^{1/2}$, where $w=(\sigma^2(F_o^2))^{-1}$. *c*) $R1=\Sigma||F_o|-|F_c||/\Sigma|F_o|$ for 1422 reflections with $I>2\ \sigma(I)$.

ment was performed assuming this absolute structure. The potassium ion was coordinated with five oxygen atoms from four carboxyl groups of three neighboring monatin molecules and one water molecule. The potassium ion also interacted with an indole ring of one of the above three monatin molecules through a π–cation interaction. As mentioned above, we successfully solved the crystal structure of the sweetest diastereomer of the monatin potassium salt dihydrate and confirmed that the absolute stereochemistry was (2*R*,4*R*).

Sweetness of (2*R*,4*R*)-Monatin Derivatives Finally, a brief structure–activity relationship study of monatin derivatives was conducted. As described above, we found that monatin and its lactone were in equilibrium in strongly acidic solutions, which gradually converted to the lactam (Fig. 5). The lactone (**9**) and lactam (**10**) of **1c** were synthesized and tasted, but neither exhibited sweetness. As expected, **9** returned to equilibrium with **1c** in water upon standing and regained its sweetness.

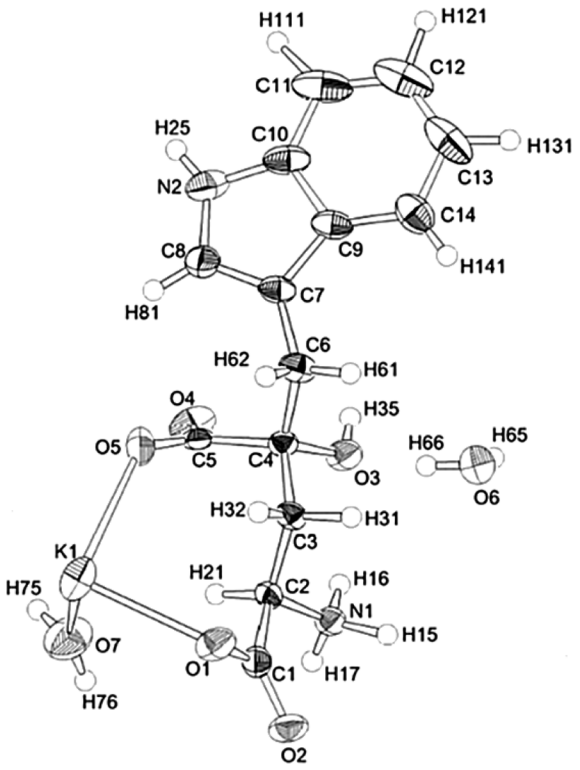


Fig. 6. ORTEP Drawing of the Molecular Structure of the (2*R*,4*R*)-Monatin Potassium Salt Dihydrate with Thermal Ellipsoids at a 50% Probability Level

The structure of monatin contains an indole-3-lactic acid fragment, in which the amino group of D-tryptophan, a sweet amino acid, is replaced by a hydroxyl group. As such, we were interested in the role of the 4-carboxyl group in sweetness expression. Thus, we synthesized derivatives of **1c** in which the 4-position carboxyl group was exchanged for other functional

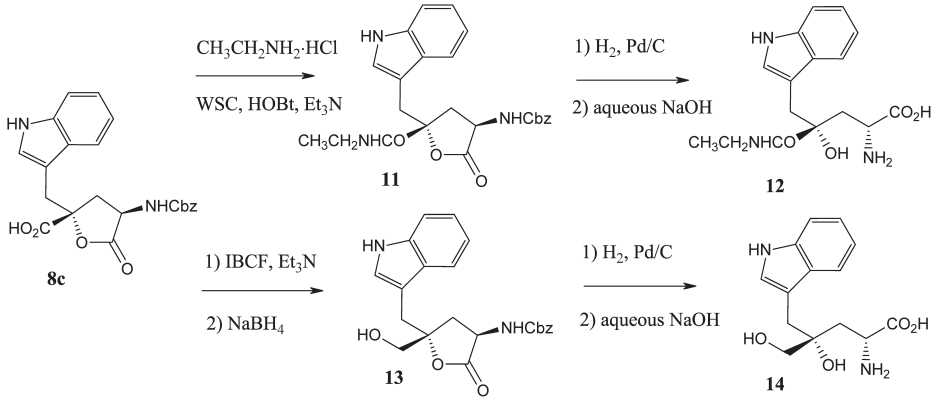


Chart 2. Synthesis of Monatin Derivatives

groups, such as ethylcarbamoyl (**12**), hydroxymethyl (**14**), and carbamoyl (relatively unstable), utilizing **8c** as the starting material, as shown in Chart 2. All derivatives were found to be as sweet as **1c**; thus, the negative charge on the 4-carboxyl group is not required for sweetness expression or for interaction with the sweetness receptor.

Conclusion

We have developed a practical racemic synthesis of monatin, in which the key intermediate, 4-hydroxy-4-(3-indolylmethyl)-2-ketoglutaric acid (**5**), was synthesized by the cross-aldol reaction of pyruvic acid and indole-3-pyruvic acid. Subsequently, the carbonyl group was converted to an amino group to afford monatin. The four stereoisomers were then isolated from the racemic monatin mixture *via* crystallization, separated from their derivatives by HPLC, and deprotected. It was found that all isomers exhibited a sweet taste, with the (2*R*,4*R*)-isomer (**1c**) being intensely sweet. However, the (2*S*,4*S*)-isomer (**1a**) was the least sweet isomer and had far lower sweetness than was previously reported. Based on these result, it was concluded that the stereoisomer (steric structure) of monatin present in nature is the (2*R*,4*R*)-isomer (**1c**), which has a significantly sweet taste. These findings will assist us in our continued investigation of the conformational requirements of novel high-potency sweeteners.

Experimental

General ^{1}H-NMR spectra were recorded on a Brucker Avance 400 spectrometer (400 MHz), and MS spectra were measured using a Thermo Quest TSQ 700 spectrometer. Amberlite IR 120B H AG was employed as the cation-exchange resin. Melting point (mp) measurements were performed using a Micro Melting Point Apparatus from Yanaco (Japan). Optical rotary power measurements were performed using a DIP-370 Digital Polarimeter manufactured by Nippon bunko (Jasco Engineerring, Japan). The chiral columns, Crownpak CR(+), CHIRALPAK AS, and CHIRALCEL OJ were purchased from DAICEL CHEMICAL INDUSTRIES, Ltd., Japan.

Preparation of Standard Samples (1) Monatin was synthesized using a modified version of the method developed by Nakamura *et al.*[6] Crude monatin (2*S*)-isomers (4.30 g) and (2*R*)-isomers (1.14 g) were adsorbed on a cation-exchange resin (H^+-type) and purified by elution with a 3% aqueous ammonia solution. Subsequent freeze-drying resulted in 2.92 g of the ammonium salts of monatin (2*S*)-isomers (a mixture of **1a**, **b**) and 711 mg of the ammonium salts of (2*R*)-isomers (a mixture of **1c**, **d**). Resolution of 660 mg of the (2*S*)-isomers and 711 mg of the (2*R*)-isomers was performed under the following preparative conditions. The fractions were neutralized with aqueous ammonia and concentrated prior to the preparative procedure. The combined fractions were adsorbed on a cation-exchange resin and eluted with an aqueous ammonia solution. The eluted fractions were freeze-dried to give 207 mg of **1a**, 233 mg of **1b**, 261 mg of **1c**, and 254 mg of **1d** as amorphous solids of their ammonium salts.

Preparative conditions for separation of monatin stereoisomers:

Guard column: Inertsil ODS-3 30×50 mm. Column: Inertsil ODS-3 30×250 mm. Detection: UV 210 nm. Eluent: ‹A› acetonitrile+0.05% trifluoroacetic acid (TFA), ‹B› H_2O+0.05% TFA. Flow rate: 28 mL/min. Gradient: 12–18% ‹A› in ‹B› over 25 min. Loaded amount: 10–13 mg. Temperature: 25°C.

Analytical conditions for monatin diastereomers:

Column: Inertsil ODS-80A 6×150 mm. Detection: UV 210 nm. Eluent: 12% $CH_3CN_{(aq)}$+0.05% TFA. Flow rate: 1.5 mL/min. Temperature: 25°C.

Analytical conditions for determination of optical purity of monatin stereoisomers:

Column: Crownpack CR(+) 4×150 mm. Detection: UV 210 nm. Eluent: aqueous perchloric acid (pH 2.0)–methanol=90 : 10. Flow rate: 1.2 mL/min. Temperature: 25°C.

Optical purity:

Values in parentheses indicate the retention time for each peak. Results are given for each stereoisomer. (2*S*,4*S*)-isomer: 94% (45.0 min); (2*S*,4*R*)-isomer: 94% (26.1 min); (2*R*,4*R*)-isomer: 94% (20.9 min); (2*R*,4*S*)-isomer: 96% (16.1 min).

Synthesis of Racemic Monatin Ammonium Salts

4-Hydroxy-4-(3-indolylmethyl)-2-ketoglutaric Acid (**5**)

Compound **2** (12.3 g, 58.7 mmol, 97% purity by weight) was dissolved in water (209 mL) containing sodium hydroxide (2.45 g). Over 2 h, a 25% aqueous sodium hydroxide solution (47.61 g) and a mixture of **3** (25.85 g, 293.5 mmol) in water (25.85 g) were added to the resulting solution under a nitrogen atmosphere at 35°C, while the reaction system was maintained at pH 11.0. Subsequently, the reaction solution was agitated for 14 h. This afforded a reaction solution containing **5** in a 44.1% yield (*vs.* **2**). Hydrochloric acid (1 N, 3.60 g) was added to neutralize the solution (275 mL, pH=6.91). A portion of the obtained reaction solution (168 mL) was passed through a resin column (4.8 cm diameter) packed with a synthetic adsorbent

(840 mL, DIAION-SP207, Mitsubishi Chemical Corporation, Japan). Ion-exchanged water was then passed through the column at a flow rate of 23.5 mL/min. Fractions eluted from 1.7 to 2.9 L were collected and concentrated to obtain **5** in a 66.3% yield.

^{1}H-NMR (D_2O) δ: 3.03 (1H, d, J=14.6 Hz), 3.11 (1H, d, J=14.6 Hz), 3.21 (1H, d, J=18.1 Hz), 3.40 (1H, d, J=18.1 Hz), 7.06–7.15 (3H, m), 7.39 (1H, d, J=7.8 Hz), 7.66 (1H, d, J=7.8 Hz). ^{13}C-NMR (D_2O) δ: 35.43, 47.91, 77.28, 109.49, 112.05, 119.44, 119.67, 121.91, 125.42, 128.41, 136.21, 169.78, 181.43, 203.58. Electrospray ionization (ESI)-MS m/z: 290.02 $(M-H)^-$.

4-Hydroxy-4-(3-indolylmethyl)-2-hydroxyiminoglutaric Acid (**6**)

Compound **2** (73.8 g, 352 mmol) was added to an aqueous sodium hydroxide solution (16 wt%, 917 g) and the resulting solution was adjusted to 35°C. An aqueous solution of **3** (50%, 310.2 g, 1761 mmol) was added portion-wise to the solution over 2 h, while the reaction solution was maintained at pH 11.1 using aqueous sodium hydroxide (30%). After an additional 4.5 h, a reaction solution containing **5** was obtained. An aqueous hydroxylamine hydrochloride salt solution (40%, 367.2 g, 2114 mmol) was then added to the reaction solution while maintaining a pH of 7 using aqueous sodium hydroxide (30%). The solution was stirred at 5°C for 17.5 h, after which the reaction solution was adjusted to pH 2 using conc. hydrochloric acid and extracted with ethyl acetate to remove the organic material. The organic layer was rinsed with aqueous saturated sodium chloride and concentrated to obtain the residue. The residue was recrystallized from aqueous ammonia (28%, 60 mL) and 2-propanol (1350 mL) to obtain the diammonium salt of **6** (142 mmol, 40% yield *vs.* **2**) as crystals.

^{1}H-NMR (D_2O) δ: 2.66 (2H, s), 2.89 (1H, d, J=14.4 Hz), 3.04 (1H, d, J=14.4 Hz), 6.89–6.94 (1H, m), 6.97–7.03 (1H, m), 7.11 (1H, d, J=2.8 Hz), 7.27 (1H, d, J=7.8 Hz), 7.53 (1H, d, J=7.8 Hz), 10.71 (1H, br s). ESI-MS m/z: 305.17 $(M-H)^-$. FAB-MS m/z: 307.0956 (M+H) (Calcd for $C_{14}H_{15}N_2O_6$: 307.0930).

Racemic Monatin Ammonium Salts (1 NH_4^+ Salt) The ammonium salt of **6** (9.6 g, 28.16 mmol) was dissolved in aqueous ammonia (28%, 180 mL) and Rh/C (5, 50% hydrous, 9.0 g) was added to the solution. The solution was stirred under a hydrogen atmosphere (10 atm., 1 MPa) at ambient temperature for 24 h. The catalyst was removed by filtration, and the filtrate was concentrated. Aqueous ethanol (90%, 80 mL) was added to the residue, and the solution was stirred at 25°C for 1.5 h. The deposited purified crystals were filtered and dried under reduced pressure to obtain racemic **1** NH_4^+ salt (7.90 g, 24.17 mmol as the diammonium salt).

(2*S*,4*S*), (2*R*,4*R*)-isomers: ^{1}H-NMR (D_2O) δ: 1.95–2.02 (1H, m), 2.58–2.62 (1H, m), 3.01–3.05 (1H, m), 3.21–3.24 (1H, m), 3.55–3.58 (1H, m), 7.07–7.11 (1H, m), 7.14–7.18 (2H, m), 7.42–7.44 (1H, d), 7.66–7.68 (1H, d).

(2*S*,4*R*), (2*R*,4*S*)-isomers: ^{1}H-NMR (D_2O) δ: 2.11–2.17 (1H, m), 2.38–2.43 (1H, m), 3.16 (2H, s), 3.90–3.93 (1H, m), 7.06–7.10 (1H, m), 7.13–7.17 (2H, m), 7.41–7.43 (1H, d), 7.66–7.68 (1H, d). ESI-MS m/z: 291.28 $(M-H)^-$.

Resolution of Mixture of Four Monatin Stereoisomer Ammonium Salts (1) into Racemic Mixture of (2*S*,4*S*)-Isomer (1a) and (2*R*,4*R*)-Isomer (1c) and Racemic Mixture of (2*S*,4*R*)-Isomer (1b) and (2*R*,4*S*)-Isomer (1d) Ammonium salts (10.00 g, 32.33 mmol) of **1** ([**1a** and **c**] : [**1b** and **d**]=60 : 40) were dissolved in an aqueous ammonia solution (2.5%, 100 mL), and the resulting solution was concentrated to 20 mL. Aqueous ammonia (5%, 3 mL) was then added, and the mixture was homogenized and allowed to stand at room temperature for 30 min. After the crystals were separated, a slurry was formed by adding an aqueous solution comprised of an aqueous ammonia solution (5%) and ethanol (80 mL, 25 : 75, v/v), and the crystals of **1a** and **c** ammonium salts were filtered. The resulting crystals were dissolved in an aqueous ammonia solution (2.5%, 30 mL), concentrated, and recrystallized from a mixed solution of aqueous ammonia (5%, 0.5 mL) and ethanol (30 mL) to give crystals of **1a** and **c** ammonium salts (4.80 g, 15.52 mmol, RP HPLC purity (hereafter referred to as "HPLC purity"): 98.0%).

^{1}H-NMR (D_2O) δ: 1.99 (1H, dd, J=11.8 and 15.3 Hz), 2.51 (1H, dd, J=2.0 and 15.2 Hz), 3.03 (1H, d, J=14.6 Hz), 3.22 (1H, d, J=14.9 Hz), 3.57 (1H, dd, J=2.0 and 11.8 Hz), 7.06–7.11 (1H, m), 7.14–7.18 (1H, m), 7.16 (1H, s), 7.42 (1H, d, J=8.1 Hz), 7.66 (1H, d, J=7.9 Hz). ESI-MS m/z: 291.39 $(M-H)^-$. mp: 182.0–186.0°C. Degree of sweetness: approximately 1300-fold (as compared to a 5% aqueous solution of sucrose).

The mother liquor ([**1a** and **c**] : [**1b** and **d**]=3 : 10) obtained in the above operation was concentrated to 5 mL. An aqueous ammonia solution (5%, 3 mL) was then added and the mixture was homogenized and allowed to stand at room temperature for 10 min. After the crystals were separated, a slurry was formed by adding ethanol (80 mL), and the crystals of **1b** and **d** ammonium salts were filtered. The resulting crystals were dissolved in an aqueous ammonia solution (2.5%, 30 mL), concentrated, and recrystallized three times from a mixed solution of aqueous ammonia (5%, 0.5 mL) and ethanol (30 mL) to give crystals of **1b** and **d** ammonium salts (3.10 g, 10.02 mmol, HPLC purity: 98.2%). The overall recovery rate was 79.0%.

^{1}H-NMR (D_2O) δ: 2.14 (1H, dd, J=10.0 and 15.3 Hz), 2.41 (1H, dd, J=2.8 and 15.3 Hz), 3.16 (2H, s), 3.92 (1H, dd, J=2.8 and 10.0 Hz), 7.06–7.10 (1H, m), 7.13–7.18 (1H, m), 7.16 (1H, s), 7.41 (1H, dd, J=0.9 and 7.9 Hz), 7.67 (1H, dd, J=1.0 and 8.0 Hz). ESI-MS m/z: 291.19 $(M-H)^-$. mp: 167.2–168.4°C. Degree of sweetness: approximately 800-fold (as compared to a 5% aqueous solution of sucrose).

Preparation of Racemic Mixture of 2-Benzyloxycarbonylamino-4-(3-indolylmethyl)-4-carboxy-γ-butyrolactone (*N*-Carbobenzoxy-γ-lactone of Monatin, Cbz-Lactone) (2*S*,4*S*)- and (2*R*,4*R*)-Isomers (8a, c) The ammonium salts of **1a** and **c** (19.51 g, 63.07 mmol; HPLC purity: 99.2%) were dissolved in a sodium hydroxide solution (2 N, 94.6 mL, 189.2 mmol) and water (90 mL). Benzyloxycarbonyl chloride (12.61 mL, 88.30 mmol) was added, and the solution was stirred for 2 h at room temperature. Additional sodium hydroxide solution (2 N, 15.8 mL, 31.54 mmol) and benzyloxycarbonyl chloride (4.50 mL, 31.54 mmol) were then added and the solution was stirred overnight at room temperature. The resulting aqueous solution was extracted with ether (50 mL, three times) to remove excess benzyloxycarbonyl chloride. The solution was adjusted to pH 3 using hydrochloric acid and extracted with ethyl acetate (100 mL, three times). The organic layer was dried over anhydrous magnesium sulfate and filtered. The filtrate was concentrated *in vacuo* to give **7a** and **c** (27.93 g, 65.5 mmol), which were subsequently dissolved in ethyl acetate (400 mL). After addition of *p*-toluenesulfonic acid (1.25 g, 6.65 mmol) the solution was heated at 75°C

for 3 h. The resulting solution was washed with water and saturated saline, dried over anhydrous magnesium sulfate, filtered, and the filtrate was concentrated *in vacuo*. Chloroform (100 mL) was then added to the residue, and the crystals were separated and collected by filtration to afford **8a** and **c** in an overall yield of 68.5% (17.64 g, 43.19 mmol; HPLC purity: 99.6%).

^{1}H-NMR (DMSO-d_6) δ: 2.39 (1H, t, J=11.3 Hz), 2.67 (1H, t, J=12.8 Hz), 3.28–3.38 (2H, m), 3.71–3.81 (1H, m), 4.98 (2H, s), 6.98 (1H, t, J=7.0 Hz), 7.07 (1H, t, J=7.0 Hz), 7.21 (1H, s), 7.25–7.35 (6H, m), 7.55 (1H, d, J=7.9 Hz), 7.67 (1H, d, J=8.3 Hz), 11.03 (1H, s). ESI-MS m/z: 409.68 $(M+H)^+$. mp: 195.5–196.9°C.

Preparation of a Racemic Mixture of Cbz-Lactone (2*S*,4*R*)- and (2*R*,4*S*)-Isomers (8b, d) The same operation described above was performed using a mixture of the **1b** and **d** ammonium salts (15.00 g, 48.49 mmol; HPLC purity: 99.5%). Heating was performed at 75°C for 2 h after addition of *p*-toluenesulfonic acid to afford **8b** and **d** in an overall yield of 61.1% (12.10 g, 29.64 mmol; HPLC purity: 100%).

^{1}H-NMR (DMSO-d_6) δ: 2.20–2.30 (1H, m), 2.60–2.70 (1H, m), 3.20 (1H, d, J=15.2 Hz), 3.42 (1H, d, J=15.1 Hz), 3.35–3.48 (1H, m), 5.03 (2H, s), 6.98 (1H, t, J=7.8 Hz), 7.05 (1H, t, J=7.8 Hz), 7.18 (1H, s), 7.28–7.40 (6H, m), 7.53 (1H, d, J=7.8 Hz), 7.80 (1H, d, J=8.5 Hz), 10.92 (1H, s). ESI-MS m/z: 409.58 $(M+H)^+$. mp: 156.7–159.1°C.

Resolution of Cbz-Lactone (2*S*,4*S*)-Isomer (8a) and Cbz-Lactone (2*R*,4*R*)-Isomer (8c) Resolution was performed using an optical isomer resolution column for **8a** and **c** (1.17 g, 2.86 mmol; HPLC purity: 99.7%). CHIRLPAK AS (20×50 mm) and CHIRALPAK AS (20×250 mm) columns were used with an eluent of *n*-hexane–ethanol–acetic acid (40 : 60 : 0.5) and a flow rate of 10 mL/min, detection at UV 210 nm, temperature of 40°C, and a loaded amount of 25 mg. The retention time was 13 min for **8a** and 23 min for **8c**. Each preparative fraction was concentrated, dissolved in ethyl acetate (50 mL), and concentrated again. The residue was crystallized from chloroform (30 mL) to afford **8a** (428 mg, 1.05 mmol) and **8c** (399 mg, 0.977 mmol) in an overall recovery yield of 70.7%. This operation was scaled up at a contract manufacturing company, providing **8a** (16.7 g) and **8c** (16.1 g) from a mixture of stereoisomers (35.6 g).

Cbz-lactone (2*S*,4*S*)-isomer (**8a**): ^{1}H-NMR spectrum is virtually identical to that of the racemic mixture of **8a** and **c**. ESI-MS m/z: 409.68 $(M+H)^+$. mp: 179.8–182.0°C. Cbz-lactone (2*R*,4*R*)-isomer (**8c**): ^{1}H-NMR spectrum is virtually identical to that of the racemic mixture of **8a** and **c**. ESI-MS m/z: 409.88 $(M+H)^+$. mp: 179.2–182.8°C.

Resolution of Cbz-Lactone (2*S*,4*R*)-Isomer (8b) and Cbz-Lactone (2*R*,4*S*)-Isomer (8d) Resolution was performed using an optical isomer resolution column for **8b** and **d** (9.89 g, 24.22 mmol; HPLC purity: 100%). CHIRALCEL OJ (20×50 mm) and CHIRALCEL OJ (20×250 mm) columns were used as the guard and preparative columns, respectively, with an eluent of *n*-hexane–ethanol–trifluoroacetic acid (40 : 60 : 0.1) and a flow rate of 8 mL/min, detection at UV 210 nm, temperature of 40°C, and a loaded amount of 50 mg. The retention time was 16 min for **8d** and 21 min for **8b**. Each preparative fraction was neutralized with aqueous ammonia and concentrated. The residue was then dissolved in ethyl acetate (150 mL), washed with an aqueous solution of hydrochloric acid adjusted to pH 3, washed with saturated saline, dried over anhydrous magnesium sulfate, and filtered, and the filtrate was concentrated *in vacuo*. The residue was crystallized with *n*-hexane (100 mL) to give a solvate of **8d** with 0.2 equiv of ethyl acetate (4.88 g, 11.45 mmol; HPLC purity: 97.3%) and a solvate of **8b** with 0.2 equiv of ethyl acetate (5.41 g, 12.70 mmol; HPLC purity: 96.9%) in an overall recovery yield of 99.7%.

Solvate of **8b**: ^{1}H-NMR spectrum is virtually identical to that of the racemic mixture of **8b** and **d**. ESI-MS m/z: 409.58 $(M+H)^+$. mp: 116.1–116.8°C. Solvate of **8d**: ^{1}H-NMR spectrum is virtually identical to that of the racemic mixture of **8b** and **d**. ESI-MS m/z: 409.58 $(M+H)^+$. mp: 109.1–110.8°C.

Conversion of Cbz-Lactone (2*R*,4*R*)-Isomer (8c) into the (2*R*,4*R*)-Isomer (1c) Sodium Salt Isomer **8c** (14.24 g, 34.85 mmol; HPLC purity: 99.5%) was dissolved in a mixed solvent of methanol (400 mL) and water (40 mL), Pd/C (10%, 3 g) was added, and reduction was performed in a hydrogen atmosphere at room temperature for 2 h. After reduction, water (100 mL) and aqueous sodium hydroxide (4 N, 19.2 mL, 76.67 mmol) were added, followed by stirring for 10 min. The catalyst was then removed by filtration and the filtrate was concentrated. In order to remove excess sodium, the residue was dissolved in water (160 mL) and an ion-exchange resin (Amberlite IR 120B H AG(H^+)) was added portion-wise until the solution became weakly acidic. Aqueous ammonia (28%, 34.8 mL) was added to the solution, and the ion-exchange resin was removed by filtration and washed with a 5% aqueous ammonia solution. The filtrate and washing solution were combined, filtered, and concentrated. The residue was dissolved in water (100 mL), activated charcoal (1 g) was added, and the solution was stirred for 10 min. The activated charcoal was removed by filtration, the filtrate was concentrated, and the concentrate was crystallized with aqueous ethanol (90%) at room temperature to give a solvate of the **1c** sodium salt with 0.2 equiv of ethanol (6.55 g, 20.19 mmol; optically active column HPLC purity: 99.3%) in an overall yield of 57.9%.

^{1}H-NMR (D_2O) δ: 2.06 (1H, dd, J=11.6 and 15.2 Hz), 2.68 (1H, dd, J=2.0 and 15.2 Hz), 3.10 (1H, d, J=14.8 Hz), 3.30 (1H, d, J=14.8 Hz), 3.64 (1H, dd, J=2.0 and 11.6 Hz), 7.16 (1H, brt, J=8.0 Hz), 7.23 (1H, brt, J=8.0 Hz), 7.24 (1H, s), 7.50 (1H, d, J=8.0 Hz), 7.74 (1H, d, J=8.0 Hz). ^{13}C-NMR (D_2O, 100 MHz) δ: 38.1, 41.0, 56.5, 83.1, 111.9, 114.5, 121.9, 122.1, 124.5, 127.8, 130.7, 138.7, 177.1, 182.9. ESI-MS m/z: 291.49 $(M-H)^-$. FAB-MS m/z: 315.0963 (M+H) (Calcd for $C_{14}H_{16}N_2NaO_5$: 315.0957). mp: 197.1–198.3°C. Specific rotation: $[\alpha]_D^{25}$ +0.64 (c=0.5, 5% NH_3). Degree of sweetness: approximately 2700-fold (as compared to a 5% aqueous solution of sucrose).

Conversion of Cbz-Lactone (2*S*,4*S*)-Isomer (8a) into the (2*S*,4*S*)-Isomer (1a) Sodium Salt The same operation described above was performed using **8a** (5.00 g, 12.25 mmol; HPLC purity: 99.8%). A solvate of the **1a** sodium salt with 0.2 equiv of ethanol (3.15 g, 9.71 mmol; optically active column HPLC purity: 99.8%) was obtained in an overall yield of 79.3%.

^{1}H- and ^{13}C-NMR spectrum are virtually identical to those of **1c**. ESI-MS m/z: 291.59 $(M-H)^-$. mp: 196.1–197.9°C. Specific rotation: $[\alpha]_D^{25}$ −1.67 (c=0.5, 5% NH_3). Degree of sweetness: approximately 50-fold (as compared to a 5% aqueous solution of sucrose).

Conversion of Cbz-Lactone (2*S*,4*R*)-Isomer (8b) into the

(2*S*,4*R*)-Isomer (1b) Sodium Salt The same operation described above was performed using **8b** with 0.2 equiv of ethyl acetate (5.23 g, 12.28 mmol; HPLC purity: 96.9%). The **1b** sodium salt (2.57 g, 8.14 mmol; optically active column HPLC purity: 99.4%) was obtained in an overall yield of 66.3%.

^{1}H-NMR (D_2O) δ: 2.21 (1H, dd, J=10.0 and 15.2 Hz), 2.48 (1H, dd, J=2.8 and 15.2 Hz), 3.23 (2H, s), 3.99 (1H, dd, J=2.8 and 10.0 Hz), 7.17 (1H, brt, J=8.0 Hz), 7.23 (1H, brt, J=8.0 Hz), 7.24 (1H, s), 7.49 (1H, d, J=8.0 Hz), 7.74 (1H, d, J=8.0 Hz). ^{13}C-NMR (D_2O, 100 MHz) δ: 36.7, 41.7, 54.8, 81.1, 112.1, 114.5, 121.9, 122.1, 124.4, 127.6, 130.7, 138.6, 177.6, 183.9. ESI-MS m/z: 291.49 $(M-H)^-$. mp: 227.1–229.4°C. Specific rotation: $[\alpha]_D^{25}$ −9.57 (c=0.5, 5% NH_3). Degree of sweetness: approximately 300-fold (as compared to a 5% aqueous solution of sucrose).

Conversion of Cbz-Lactone (2*R*,4*S*)-Isomer (8d) into the (2*R*,4*S*)-Isomer (1d) Sodium Salt The same operation described above was performed using **8d** with 0.2 equiv of ethyl acetate (3.66 g, 8.59 mmol; HPLC purity: 97.3%). The **1d** sodium salt (2.23 g, 7.07 mmol; optically active column HPLC purity: 99.2%) was obtained in an overall yield of 82.3%.

^{1}H- and ^{13}C-NMR spectrum are virtually identical to those of **1b**. ESI-MS m/z: 291.19 $(M-H)^-$. mp: 227.5–229.2°C. Specific rotation: $[\alpha]_D^{25}$ +11.08 (c=0.5, 5% NH_3). Degree of sweetness: approximately 1300-fold (as compared to a 5% aqueous solution of sucrose).

Synthesis of (2*R*,4*R*)-2-Amino-4-hydroxy-4-ethylcarbamoyl-4-(3-indolylmethyl)butyric Acid (12) Isomer **8c** (817 mg, 2.00 mmol) was dissolved in a mixed solvent containing tetrahydrofuran (10 mL), dichloromethane (10 mL), and dimethylformamide (10 mL) maintained at a temperature of 0°C. To this solution, ethylamine hydrochloride (244 mg, 3.0 mmol), triethylamine (0.56 mL, 4.0 mmol), 1-hydroxybenzotriazole hydrate (324 mg, 2.40 mmol), and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (463 mg, 2.41 mmol) were added, and the solution was stirred overnight at room temperature. The reaction solution was then concentrated *in vacuo* and water (50 mL) was added to the residue. The solution was extracted with ethyl acetate (50 mL, twice), and the organic layer was washed with a 5% aqueous citric acid solution (30 mL, twice), a saturated sodium chloride solution (30 mL, once), a 5% aqueous sodium bicarbonate solution (30 mL, twice), and a saturated sodium chloride solution (30 mL, once). The organic layer was dried over anhydrous magnesium sulfate, filtered, and the filtrate was concentrated *in vacuo*. The residue was purified by preparative thin-layer chromatography (PTLC), affording (2*R*,4*R*)-2-benzyloxycarbonylamino-4-ethylcarbamoyl-4-(3-indolylmethyl)-γ-butyrolactone (**11**, 688 mg, 1.58 mmol).

Compound **11** (688 mg, 1.58 mmol) was then dissolved in methanol (10 mL), and Pd/C (10%, 317 mg) was added to the solution. Reduction was performed at room temperature in a hydrogen atmosphere at atmospheric pressure for 16 h. After removing the catalyst by filtration, the reaction solution was concentrated *in vacuo*. The residue was dissolved in tetrahydrofuran (15 mL) and a sodium hydroxide solution (2 N, 1.5 mL) was added, followed by stirring for 10 min. The reaction solution was concentrated *in vacuo* and the residue was dissolved in water (5 mL). The solution was neutralized with a strongly acidic ion-exchange resin (Amberlite IR120B H AG). Ethanol was then added to the solution and the deposited crystals were filtered, affording **12** (244 mg, 7.60 mmol) as crystals.

^{1}H-NMR (CD_3OD) δ: 0.88 (3H, t, J=7.2 Hz), 2.12 (1H, dd, J=9.4 and 15.0 Hz), 2.63 (1H, dd, J=4.2 and 15.0 Hz), 3.05–3.17 (2H, m), 3.17 (1H, d, J=14.5 Hz), 3.30 (1H, d, J=14.5 Hz), 3.68 (1H, dd, J=4.1 and 9.3 Hz), 6.90–7.10 (1H, m), 7.08 (1H, t, J=7.2 Hz), 7.13 (1H, s), 7.32 (1H, d, J=8.4 Hz), 7.62 (1H, d, J=7.8 Hz). ESI-MS m/z: 320.21 $(M+H)^+$, 318.21 $(M-H)^-$. FAB-MS m/z: 320.1617 (M+H) (Calcd for $C_{16}H_{22}N_3O_4$: 320.1610).

Synthesis of (2*R*,4*R*)-2-Amino-4,5-dihydroxy-4-(3-indolylmethyl)pentanoic Acid (14) Isomer **8c** (1.02 g, 2.50 mmol) and triethylamine (0.52 mL, 3.75 mmol) were dissolved in tetrahydrofuran (7.5 mL). The reaction solution was cooled to between −15 and −40°C, and isobutyl chloroformate (0.51 mL, 3.75 mmol) was added to the solution. After stirring at −15 to −40°C for 30 min, a solution of sodium borohydride (314 mg, 8.30 mmol) dissolved in water (1.5 mL) was added, and the solution was stirred for an additional 20 min. After adding hydrochloric acid (2 N, 2.5 mL), the solution was concentrated *in vacuo*. The residue was dissolved in water (10 mL) and extracted with ethyl acetate (25 mL, twice). Once the resulting organic layer was washed with water (10 mL) and a saturated sodium chloride solution (10 mL), it was dried over anhydrous magnesium sulfate, filtered, and the filtrate was concentrated *in vacuo*. The residue was purified by PTLC, affording (2*R*,4*R*)-2-benzyloxycarbonylamino-4-hydroxymethyl-4-(3-indolylmethyl)-γ-butyrolactone (**13**, 880 mg, 2.23 mmol) as a viscous, oily product.

Compound **13** (880 mg, 2.23 mmol) was then dissolved in methanol (10 mL) and Pd/C (10%, containing 50% water, 410 mg) was added. After reduction at room temperature under a hydrogen atmosphere at atmospheric pressure for 5 h, sodium hydroxide (1 N, 2.15 mL) was added. The catalyst was removed by filtration, and the filtrate was concentrated *in vacuo*. The residue was dissolved in water (15 mL) and tetrahydrofuran (15 mL), and the solution was neutralized with a strongly acidic ion-exchange resin (Amberlite IR120B H AG). The resin was removed by filtration, and the filtrate was freeze-dried, affording **14** (228 mg, 0.82 mmol) as a powder.

^{1}H-NMR (CD_3OD) δ: 1.84 (1H, dd, J=10.8 and 15.2 Hz), 2.27 (1H, dd, J=2.8 and 15.6 Hz), 2.97 (1H, d, J=14.4 Hz), 3.06 (1H, d, J=1.48 Hz), 3.56 (1H, d, J=11.2 Hz), 3.67 (1H, d, J=11.6 Hz), 3.87 (1H, dd, J=2.8 and 10.8 Hz), 7.01 (1H, t, J=8.0 Hz), 7.10 (1H, t, J=8.0 Hz), 7.15 (1H, s), 7.34 (1H, dd, J=8.0 Hz), 7.64 (1H, d, J=8.0 Hz). ESI-MS m/z: 265.30 $(M+H)^+$, 263.10 $(M-H)^-$. FAB-MS m/z: 279.1358 (M+H) (Calcd for $C_{14}H_{18}N_2O_4$: 279.1345).

Synthesis of (2*R*,4*R*)-2-Amino-2,3-dideoxy-4-*C*-(1*H*-indol-3-ylmethyl)pentaric Acid 1,4-Lactone (9, Monatin Lactone) Isomer **8c** (2.0 g, 4.90 mmol) was dissolved in a mixed solvent of tetrahydrofuran (60 mL) and water (10 mL). To this solution was added Pd/C (10%, 1.0 g), and the pH was adjusted to slightly basic by adding aqueous ammonia (28%). Reduction was performed in a hydrogen atmosphere at room temperature for 4 h. After reduction, the catalyst was removed by filtration, and the filtrate was concentrated. The residue was crystallized with ethyl ether and then the obtained crystals were recrystallized from a small amount of water to give **9** (0.76 g, 2.76 mmol).

^{1}H-NMR (D_2O) δ: 2.38 (1H, dd, J=10.8 and 13.6 Hz), 2.92

(1H, dd, J=9.8 and 13.6 Hz), 3.17 (1H, t, J=10.6 Hz), 3.37 (2H, s), 7.16 (1H, t, J=6.9 Hz), 7.18 (1H, t, J=8.2 Hz), 7.27 (1H, s), 7.46 (1H, d, J=8.0 Hz), 7.68 (1H, d, J=8.0 Hz). ESI-MS m/z: 275.51 $(M+H)^+$.

Synthesis of (2*R*,4*R*)-4-Hydroxy-4-(1*H*-indol-3-ylmethyl)-5-oxo-proline (10, Monatin Lactam) The **1c** sodium salt (3.00 g, 9.05 mmol) was dissolved in aqueous phosphoric acid (pH 2.0, 120 mL), and the solution was heated at 90°C for 4 d. The solution was concentrated to 10 mL and extracted with ethyl acetate (100 mL, three times). The organic solution was washed with water (50 mL), dried over anhydrous magnesium sulfate, filtered, and concentrated *in vacuo*. The residue was recrystallized from chloroform, affording **10** (1.56 g, 5.70 mmol) as crystals.

^{1}H-NMR (DMSO-d_6) δ: 1.80 (1H, dd, J=6.6 and 13.0 Hz), 2.41 (1H, dd, J=8.2 and 13.0 Hz), 2.89 (1H, d, J=14.1 Hz), 2.97 (1H, d, J=14.1 Hz), 3.46 (1H, t, J=7.9 Hz), 5.45 (1H, br s), 6.93–6.98 (1H, m), 7.04 (1H, t, J=7.0 Hz), 7.16 (1H, d, J=2.3 Hz), 7.32 (1H, d, J=8.0 Hz), 7.62 (1H, d, J=7.9 Hz), 7.97 (1H, s), 10.88 (1H, br s). ESI-MS m/z: 275.06 $(M+H)^+$.

Preparation of (2*R*,4*R*)-Monatin Potassium Salt Dihydrate Crystals for X-Ray Crystal Structure Analysis The **1c** ammonium salt (1.5 g) was dissolved in water (10 mL), and the solution was passed through a column filled with a cation-exchange resin (25 mL, Diaion PK 228, potassium-type, Mitubishi Chemical). The resulting eluent was concentrated to 11.5 g and dissolved in ethanol (60 mL) at 60°C. The solution was cooled to 10°C and stirred overnight. Precipitated crystals were collected by filtration and dried to give the **1c** potassium salt (1.1 g, mp: 213–214°C).

The **1c** potassium salt (200 mg) was then dissolved in water (2.0 mL) and heated to 65°C. Ethanol (10 mL) was added, and the solution was cooled to room temperature. A small amount of seed crystal was added to the solution and allowed to stand until the crystal grew. Approximately 1 month later, the mother liquor was removed and the crystal was dried.

Single Crystal X-Ray Studies X-Ray diffraction data of a crystal of the **1c** potassium salt dihydrate were collected using a Rigaku AFC5S diffractometer operated at 40 kV and 30 mA, with graphite monochromated Cu$K\alpha$ radiation (λ=1.54178 Å). Cell constants were obtained from a least-squares refinement using the setting angles of 24 carefully centered reflections. The data were collected at a temperature of 296±1 K using the ω–2θ scan technique to a maximum 2θ value of 159.4°. The linear absorption coefficient (μ) for the Cu$K\alpha$ radiation was 31.1 cm^{-1}. An empirical absorption correction based on azimuthal scans of several reflections was applied, which resulted in transmission factors ranging from 0.86 to 1.00. A correction for the secondary extinction was applied (coefficient=1.59514e-06). The crystal structure was solved by direct methods[32] and expanded using Fourier techniques.[33] In the full-matrix least-squares refinement, non-hydrogen atoms and hydrogen atoms were refined anisotropically and isotropically, respectively. Anomalous dispersion effects were included in the F_{calc}. All calculations were performed using the teXsan[34] crystallographic software package from Molecular Structure Corporation.

Sensory Evaluation The sweetness intensity of each stereoisomer of monatin was determined relative to sucrose in water by the following two methods.

[Method 1] An aqueous solution of each test compound was prepared without any pH adjustment. Subjects, who were members of our research group, tested each solution maintained at room temperature with a "sip and spit" method. They diluted each solution with water until they perceived the same sweetness intensity as a 5% sucrose solution. The sweetness potency was calculated by dividing the concentration of 5% sucrose by the concentration that was perceived to have equal sweetness.

[Method 2] An aqueous solution of each test compound nearly equal to 10% sucrose was prepared according to the above results (solution A). Solutions of 7.56% (score 1), 8.76% (score 2), 10.00% (score 3), 11.50% (score 4), and 13.23% (score 5) sucrose were prepared as external standard solutions (solution B). Seven trained panelists scored each test sample by comparing the sweetness intensity of solutions A and B. The point of sucrose equivalency (Y) of solution A was calculated by an approximate equation (Y=6.575e$^{0.1398X}$) and the average score of each test compound (X). The sweetness potency was then calculated by dividing Y by the concentration of the test compound.

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Conflict of Interest All authors were employees of Ajinomoto Co., Inc. when this study was conducted and have no further conflicts of interest to declare.

References

1) Vleggaar R., Ackerman L. G. J., Steyn P. S., *J. Chem. Soc., Perkin Trans. 1*, **1992**, 3095–3098 (1992).
2) Van Wyk P. J., Ackerman L. G., U.S. Patent 4975298 (1990)
3) Van Wyk P. J., Ackerman L. G., U.S. Patent 5128164 (1992).
4) Holzapfel C. W., Bischofberger K., Olivier J., *Synth. Commun.*, **24**, 3197–3211 (1994).
5) Abushanab E., Arumugam S., US Patent 5994559 (1999).
6) Nakamura K., Baker T. J., Goodman M., *Org. Lett.*, **2**, 2967–2970 (2000).
7) Fujiyasu J., Watanabe H., Kitahara T., Abstract of Papers Presented at the General Meeting in 2000 of the Agricultural Chemical Society of Japan, 3B128β (page 221).
8) Kitahara T., Watanabe H., JP Patent 2002060382A2 (2002).
9) Tamura O., Shiro T., Toyao A., Ishibashi H., *Chem. Commun.*, **2003**, 2678–2679 (2003).
10) Oliveira D. J., Coelho F., *Tetrahedron Lett.*, **42**, 679–3796 (2001).
11) Tamura O., Shiro T., Ogasawara M., Toyao A., Ishibashi H., *J. Org. Chem.*, **70**, 4569–4577 (2005).
12) Bassoli A., Borgonovo G., Busnelli G., Morini G., Drew M. G. B., *Eur. J. Org. Chem.*, **2005**, 1652–1658 (2005).
13) Schiffman S. S., Dackis C., *Percept. Psychophys.*, **17**, 140–146 (1975).
14) Shallenberger R. S., Acree T. E., Lee C. Y., *Nature* (London), **221**, 555–556 (1969).
15) Helaine V., Bolte J., *Tetrahedron Asymmetry*, **9**, 3855–3861 (1998).
16) Kasai T., Nishitoba T., Shiroshita Y., Sakamura S., *Agric. Biol. Chem.*, **48**, 2271–2278 (1984).

17) Larsen P. O., Sørensen H., Cochran D. W., Hagaman E. W., Wenkert E., *Phytochemistry*, **12**, 1713–1719 (1973).
18) Olsen O., Sørensen H., *Phytochemistry*, **19**, 1717–1721 (1980).
19) Rhugenda-Banga N., Welter A., Jadot J., Casimir J., *Phytochemistry*, **18**, 482–484 (1979).
20) Juhl K., Gathergood N., Jørgensen K. A., *Chem. Commun.*, **2000**, 2211–2212 (2000).
21) Gathergood N., Juhl K., Poulsen T. B., Thordrup K., Jørgensen K. A., *Org. Biomol. Chem.*, **2**, 1077–1085 (2004).
22) Wiley R. H., Kim K.-S., *J. Org. Chem.*, **38**, 3582–3585 (1973).
23) Leussing D., Stanfield C. K., *J. Am. Chem. Soc.*, **86**, 2805–2810 (1964).
24) Margolis S. A., Coxon B., *Anal. Chem.*, **58**, 2504–2510 (1986).
25) Passerat N., Bolte J., *Tetrahedron Lett.*, **28**, 1277–1280 (1987).
26) Ruffo A., Testa E., Adinolfi A., Pelizza G., *Biochem. J.*, **85**, 588–593 (1962).
27) Kawahara S., Amino Y., Mori K., Funakoshi N., Takemoto T., WO Patent 2003059865A1 (2003).
28) Amino Y., Yuzawa K., Mori K., Takemoto T., WO Patent 2003045914A1 (2003).
29) Fry J. C., Yurttas N., Biermann K. L., Lindley M. G., Goulson M. J., *J. Food Sci.*, **77**, S362–S364 (2012).
30) Ariyoshi Y., *Agric. Biol. Chem.*, **40**, 983–992 (1976).
31) Flack H. D., *Acta Crystallogr. A*, **39**, 876–881 (1983).
32) Fan H.-F., SAPI91: Structure Analysis Programs with Intelligent Control, Rigaku Corporation, Tokyo, Japan, 1991.
33) Beurskens P. T., Admiraal G., Beurskens G., Bosman W. P., de Gelder R., Israel R., Smits J. M. M., DIRDIF94: The DIRDIF-94 program system, Technical Report of the Crystallography Laboratory, University of Nijmegen, the Netherlands, 1994.
34) teXsan: Crystal Structure Analysis Package, Molecular Structure Corporation, 1985 & 1999.