Structural Elucidation of 4-(Cystein-S-yl)butyl Glucosinolate from the Leaves of *Eruca sativa*

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The structurally unique glucosinolate (GSL), 4-(cystein-S-yl)butyl GSL, was identified in the leaves of hydroponically-grown rocket salad (*Eruca sativa* Mill.). Its electrospray ionization mass spectrometry (ESI-MS)/MS spectrum indicated that this unusual GSL had a molecular weight of 414 as a desulfo (DS)-GSL, and a molecular formula of C_{14}H_{25}N_{2}O_{8}S_{2} based on its negative ion matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) spectrum. For further confirmation, the 4-(cystein-S-yl)butyl DS-GSL was prepared with authentic L-Ser and purified dimeric 4-mercaptobutyl DS-GSL, and its chemical structure then confirmed by ESI-MS/MS data. It is named “glucorucolamine” as a trivial name from its ammonia sensitivity. This unique GSL was found to the greatest extent when rocket salad was grown in a 100% NH_{4}^{+}-N nutrient solution. Despite it clearly seems to reduce the detoxification of excess NH_{4}^{+} in the leaves of rocket salad, present knowledge about the unique GSL is still far from being sufficient.

**Key words:** ammonia detoxification; electrospray ionization mass spectrometry (ESI-MS)/MS; 4-mercaptobutyl glucosinolate; L-Ser; rocket salad (*Eruca sativa* Mill.)

Given its spicy hot taste and short biological cycle (45–60 days), the economic potential of the cultivated annual herb ‘rocket salad’ [*Eruca sativa* Mill.; *Brassicaceae*] has risen steadily in Japan since the 1990s.1 In Europe, it is usually consumed as a leafy vegetable, whereas in India and Pakistan it is grown for its oil seed production.2 There are mainly two species of rocket: *Eruca sativa* Mill. (also known as garden rocket or rucola) and *Diplotaxis tenuifolia* L. (wild rocket).3 Cultivated rocket is characterized by its rough, stiff, branched stems and highly irregular; alternate, fleshy and somewhat hairy, 50–150 mm long leaves occurring in rosettes.2,4,5 Rocket is rich in fiber, iron and particularly in vitamins A and C.3 When crushed, the leaves emit a strong odor of sesame seeds.4,5 It is thought that the characteristic pungent or bitter flavor of rocket salad may be derived from the presence of glucosinolates (GSLs) and their breakdown products, particularly 4-mercaptobutyl isothiocyanate (ITC).5 These are known to contribute to the characteristic unpalatable flavor of *Brassica* crops and to exhibit several biological activities, including anti-carcinogenic, anti-fungal and antibacterial effects.7

Approximately 120 individual GSLs have been isolated, mainly from the *Brassicaceae* family.8 They are distinguished from one another by a variable R group (see Table 1) derived from one or several amino acids including alanine, valine, leucine, isoleucine, phenylalanine, tyrosine and tryptophan.9 They are stored in the vacuoles of plant cells, but following cell damage and exposure to myrosinase, a thioglucosidase, they are hydrolyzed to form isothiocyanates which have garnered great interest for their potential role in promoting human health10 and other products11 since the 1990s.

Elucidated by the oxidation of 4-mercaptobutyl ITC, a prominent component of the dichloromethane extract of rocket salad analyzed for pesticide residues was identified by gas chromatography (GC), GC–mass spectrometry (GC–MS) and nuclear magnetic resonance (NMR) techniques to be bis(4-isothiocyanatobutyl)

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**Abbreviations:** ESI-MS, electrospray ionization mass spectrometry; GSL, glucosinolate; HPLC, high-performance liquid chromatography; MALDI-TOF-MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; NMR, nuclear magnetic resonance spectroscopy
disulfide, a GSL breakdown product.\(^\text{12}^\) The precursor of this ITC was later identified as 4-mercaptobutyl GSL, together with the corresponding dimeric 4-mercaptobutyl GSL.\(^\text{6}^\) The authors concluded that the unusual disulfide, as well as the corresponding dimeric 4-mercaptobutyl GSL, were artifacts derived from 4-mercaptobutyl GSL by non-enzymatic oxidation during the extraction process. Derived from the pathways of mercaptobutyl GSL by non-enzymatic oxidation during the extraction process. Derived from the pathways of mercaptobutyl GSL, were artifacts derived from 4-disulfide, as well as the corresponding dimeric 4-disulfide, a GSL breakdown product.\(^\text{12}^\) The precursor of this ITC was later identified as 4-mercaptobutyl GSL, together with the corresponding dimeric 4-mercaptobutyl GSL.\(^\text{6}^\) The authors concluded that the unusual disulfide, as well as the corresponding dimeric 4-mercaptobutyl GSL, were artifacts derived from 4-mercaptobutyl GSL by non-enzymatic oxidation during the extraction process. Derived from the pathways of GSL biosynthesis and hydrolysis reactions within the chain elongation cycle of 1-methionine, glucorucin, the most prominent GSL in the seeds and leaves of *E. sativa*, served as a precursor in the formation of glucoraphanin (by S-oxidation) and 4-mercaptobutyl GSL (by S-demethylation). This was followed by the formation of dimeric 4-mercaptobutyl GSL by non-enzymatic oxidation.\(^\text{6}^\) Furthermore, Kim *et al.*\(^\text{1}^\) have discovered the structurally unique GSL, 4-(β-o-glucopyranosyldisulfanyl)butyl GSL, in rocket leaves. We have recently reported the presence of another unknown GSL compound whose presence, along with that of the indolyl GSLs (glucoraphanin and 4-methoxyglucobrassicin), was linked to conditions of an excessive NH\(_4^+\)-N nutrient level.\(^\text{13}^\) It had a relatively high molecular weight (M\(_s\)) of 414 as DS-GSL obtained by the MS data. The unknown GSL did not match any GSL compound in our data base of high-pressure liquid chromatography (HPLC) profiles and MS data, even though its retention time (t\(_R\)) by HPLC was very close to that of 4-methylsulfinylobutyl GSL (trivial name of glucoraphanin, M\(_s\) of 357 as DS-GSL), one of the major GSLs in rocket salad.\(^\text{14}^\)

Several methods have been developed for the quantitative and qualitative analysis of intact GSLs, DS-GSLs and their breakdown products.\(^\text{15}^\) Amongst these, the determination of individual GSLs by HPLC of DS-GSLs generated by aryl sulfatase has been established as the official reference method of the European Community since 1990 and of the International Standardization Organization (ISO) since 1992.\(^\text{16}^\) Separation of DS-GSLs by HPLC is easier than that of the native forms, and contamination tends to be lessened because the process of desulfation typically leads to a cleaner sample.\(^\text{15}^\) Therefore, in this present study, only desulfated GSLs were subjected to chemical analyses. The work described here was to isolate the unknown GSL compound by HPLC and to elucidate its structure by means of ESI-MS/MS, MALDI-TOF-MS and NMR techniques.

**Materials and Methods**

*Chemicals.* HPLC-grade acetonitrile (CH\(_3\)CN), methanol (MeOH), L-Ser and ODS C18 matrix (Wako 40C18, 30–50-μm particles) for open-column chromatography were purchased from Wako Pure Chemical Industries (Osaka, Japan). Aryl sulfatase (type H-1, EC 3.1.6.1) and 2-mercaptoethanol were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). DEAE-Sephadex A-25 was supplied by Amersham Biosciences (Uppsala, Sweden).

*Plant material.* Rocket seeds (cultivar Odyssey) were purchased from Sakata Seed Co. (Yokohama, Japan). The seeds were sown in a cell pot containing vermiculite, and the seedlings transferred to a wooden container (0.9 × 1.8 × 0.1 m) about 14 days after sowing (DAS). The plantlets were grown in a glasshouse at the National Agricultural Research Center for the Hokkaido Region (Memuro, Hokkaido; longitude, 143°03'E; latitude, 42°55'N). The temperature in the glasshouse was maintained above 25°C by a boiler system, and natural light periods were reinforced for 4h (16:00–20:00) with four 500-W fluorescent lamps, positioned 1.8 m from the plants (mean 27 μmol m\(^{-2}\) s\(^{-1}\)) the plants were nourished with Hoagland-type\(^\text{17}^\) solutions as follows [macro-nutrients: KCl 6 mmol l\(^{-1}\), (NH\(_4\))\(_2\)SO\(_4\) 1 mmol l\(^{-1}\), (NH\(_4\))\(_2\)PO\(_4\)+3H\(_2\)O 1 mmol l\(^{-1}\), CaCl\(_2\) 2 mmol l\(^{-1}\), MgCl\(_2\) 1 mmol l\(^{-1}\), NH\(_4\)Cl 5 mmol l\(^{-1}\) for 100% NH\(_4^+\)-N and KCl 3 mmol l\(^{-1}\), Ca(NO\(_3\))\(_2\) 2 mmol l\(^{-1}\), KNO\(_3\) 1 mmol l\(^{-1}\), NH\(_4\)NO\(_3\) 2.5 mmol l\(^{-1}\), MgSO\(_4\) 1 mmol l\(^{-1}\),...
K₂HPO₄ 1 mmol l⁻¹ for 25:75% (NH₄)₂SO₄; Na₂CO₃; NaCl; and 1 ml of a 0.02 M acetate buffer (pH 5.0). The total N content of the solution was 10 mm, entirely in the (NH₄)₂SO₄ form.⁵

Rocket salted leaves were harvested and immediately stored at −20°C. Samples then being lyophilized, ground to a fine powder and stored in a desiccator until needed for chemical analysis. The process just described was replicated several times during the 2004 seasons to collect sufficient rocket leaves for the macro-extraction of GSLs.

Preparation of the crude extract and DS-GSLs. A 100-mg aliquot of the freeze-dried powder was placed in a 2.0-ml microcentrifuge tube, and 1.5 ml of boiling 70% (v/v) aqueous MeOH was added to inactivate the endogenous myrosinase activity. After swirling, the sample was heated for 5 min in a water bath preheated to 70°C. An aliquot (100 µl) of sinigrin (1 mg ml⁻¹) was then added as an internal standard. The mixture was centrifuged (13,000 g, 10 min, 4°C) and the resulting supernatant collected. The residue was re-extracted with boiling 70% (v/v) aqueous MeOH twice more, and the combined supernatants were taken as the crude GSL extract.⁶

This crude extract was then put into a DEAE-Sephadex A-25 (40 mg dry wt.) mini-column housed in a 1,000-µl pipet tip which was washed twice with 1 ml of de-ionized water and 1 ml of a 0.02 M acetate buffer (pH 5.0). The DS-GSLs were released by adding 75 µl (29 units) of an aryl sulfatase solution (23 mg ml⁻¹) to the mini-column. The column was closed at both ends with paraffin film and allowed to stand for about 16 h at ambient temperature. The DS-GSLs were eluted into a 2.0-ml microcentrifuge tube with 0.5 ml (x 4) of de-ionized water.⁶

HPLC analysis. Separation of DS-GSL was carried out in a reversed-phase Inertsil ODS-2 column (250 × 4.6 mm i.d., 5 µm; GL Sciences, Tokyo, Japan) with an E-type cartridge guard column (10 × 4.0 mm i.d., 5 µm), using 1100 series HPLC apparatus (Agilent Technologies, CA, USA). The column was set at 35°C, and a flow rate of 1.0 ml min⁻¹ and detection wavelength of 227 nm were used. The elution solvents were de-ionized water (solvent A) and 20% aqueous (v/v) CH₃CN (solvent B). The samples were eluted according to the following gradient: a linear increase from 1% to 99% of solvent B in 18 min, fixed 99% of solvent B for the next 11 min and a linear decrease from 99% to 1% of solvent B in 3 min.²¹ Aliquots of 20 µl were injected with the Agilent G1329A automatic injector.

LC-ESI-MS/MS, MALDI-TOF-MS and NMR analyses. For the unknown GSL compound, the MS data were collected with an LC-ESI-MS/MS system consisting of Agilent 1100 series HPLC apparatus coupled to an Esquire 3000-plus ion trap mass spectrometer (Bruker Daltonics, MA, USA) operated in the positive mode. Both the auxiliary and the sheath gases were nitrogen at a flow rate of 12 liter min⁻¹. The drying gas temperature was set at 365°C, and the nebulizer pressure, at 70 psi. The capillary voltage was 4.5 kV. Spectra were recorded in the positive-ion mode between m/z 100 and 800. The Esquire 3000 plus instrument was used with Agilent Chemstation software for data acquisition and evaluation.

A MALDI-TOF-MS analysis of the isolated unknown compound was performed in the negative-ion mode with a Voyager-DE STR-H (Applied Biosystems, Foster, CA, USA) operated at a −20-kV accelerating voltage, a pulse delay time of 145 ms and a grid voltage of 67%. Internal calibration was performed using 3-aminonquinoline (matrix: [M − H⁻]⁻, 143,0604) and 5, 10, 15, 20-tetraphenyl-21H, 23H-porphine ([M⁺]⁺, 614.2465) as calibrants.

NMR spectra were recorded on a Jeol ECA-500 spectrometer (¹H-NMR at 500 MHz and ¹³C-NMR at 126 MHz), with a JMS-SX102A mass spectrometer (Jeol USA, Peabody, MA, USA). D₂O for ¹H-NMR and CDCl₃ for ¹³C-NMR were used as standards at 4.65 ppm and 77.0 ppm, respectively.

Macro-extraction of DS-GSLs and purification of the unknown DS-GSL. This process was a slightly modified version of the method reported by Kim et al.¹ Briefly, crude GSLs were extracted with boiling 80% (v/v) MeOH from lyophilized rocket leaves. The extracted GSLs were converted to DS-GSLs in a column (45 × 2.5 cm i.d.) packed with DEAE-Sephadex A-25 (H⁺) and desulfated by the addition of an aryl sulfatase solution (23 mg/ml, 8,120 units). The unknown DS-GSL was isolated from the mixed DS-GSLs by using a 40C₁₈ ODS open column (45 × 4.1 cm i.d.) with 10% (v/v) aqueous CH₃CN and then a 40C₁₈ ODS open column (95 × 1.5 cm i.d.) with 2% (v/v) aqueous CH₃CN. The unknown DS-GSL was purified in an STR ODS-II column (250 × 15 mm i.d., 5 µm; Shinwa Chemical Industries Ltd., Kyoto, Japan) and then in an Inertsil ODS-2 column (250 × 4.6 mm i.d., 5 µm) with the Agilent 1100 series HPLC apparatus. It was necessary to perform several runs of the latter process to collect a sufficient amount of each pure GSL because a relatively high concentration and high-purity of the sample were required for the NMR analysis of each HPLC-isolated GSL.²²

Consequently, ca. 0.9 mg dry wt. of the unknown GSL compound was obtained from 2.1 kg of freeze-dried powder. The MALDI-TOF-MS data gave the following results: m/z 413.10291 [M − H⁻]⁻ (calc. for C₁₂H₂₀O₆N₂S₂, 413.10469).¹³C-NMR (500 MHz, D₂O) δₚ: 4.83 (1H, d, J = 9.4 Hz, H-1), 3.74 (1H, dd, J = 4.3, 7.4 Hz, H-2), 3.73 (1H, dd, J = 1.6, 12.9 Hz, H-6a),

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Determination of free amino acids. Harvested rocket leaves (ca. 100 mg dry wt.) were homogenized in 5 ml of 80% (v/v) ethanol for 1 min, and the homogenate was allowed to stand at ambient temperature for 1 h. After filtering through two layers of No. 2 filter paper by using an aspirator, the filtrate was concentrated at 37°C. The mixture, 2 mg of an L-Ser solution in 500 μl of 2-mercaptoethanol, and the mixture was allowed to stand alone for 2 days after stirring for 3 min. To the mixture, 2 mg of an L-Ser solution in 500 μl of H2O and then 150 μl (50 μl × 3) of 0.1 N NaOH solutions were added (pH 8.3 and pH 9.3, respectively). After stirring the mixture for 3 min, the pH value was finally adjusted to 6.9–7.0 by the addition of approximately 200 μl (50 μl × 4) of a 2 N HCl solution. The final total volume was 1.35 ml. After the replacement reaction, the solution was applied to LC-ESI-MS/MS, and several total ion chromatograms and their MS/MS data were obtained. The compounds at retention times (tR) 20.7, 2.9, 3.9 and 14.1 min of the chromatograms were identified as 2 (ions at m/z 653 [M + H]+), 5 (m/z 106 [M + H]+), 1 (m/z 415 [M + H]+) and 4 (m/z 328 [M + H]+), respectively.

### Results and Discussion

#### Identification of DS-GSLs by ESI-MS/MS

An unknown GSL has been detected from the leaves and roots of rocket salad when plants were cultivated in a 100% NH4+-N nutrition solution.13 It was present together with two indolyl GSLs: indol-3-ylmethyl GSL and 4-methoxyindol-3-ylmethyl GSL. These indolyl GSLs had already been identified in rocket leaves.14 To structurally elucidate the unknown GSL compound, rocket salad was cultivated and collected from a soil-less culture with a 100% NH4+-N nutrition solution during the 2004 season. The freeze-dried leaf powder was extracted with boiling 80% (v/v) aqueous MeOH to obtain crude GSLs. The boiling 80% MeOH extracts were purified in a DEAE-Sephadex A-25 column (H+ type) after desulfating the GSLs with aryl sulfatase, since the process of GSL desulfation typically leads to cleaner samples.15 The separation and identification of different DS-GSLs were performed by LC-ESI-MS/MS. As indicated by the HPLC profiles (data not shown), several peaks were obtained. These DS-GSLs were identified (Table 1)13 as follows: no. II, 4-methylsulfinylbutyl DS-GSL (trivial name, glucoraphanin); III, 4β,6-glucopyranosylsulfanyl)butyl DS-GSL (“diglucothiobienin”); IV, 4-methythiobutyl DS-GSL (glucoercycin); V, indol-3-ylmethyl DS-GSL (glucobrassicin); VI, dimeric 4-mercaptopbutyl DS-GSL; VII, 4-methoxyindol-3-ylmethyl DS-GSL (4-methoxyglucobrassicin). The DS-GSL corresponding to I has not previously been identified in rocket leaves.6,12-14

#### Structural elucidation of the unknown GSL

Unknown GSL I was further purified by column chromatography after freeze-drying of the eluate, and its identity confirmed by the retention time on the HPLC profile and its ESI-MS/MS data. The unknown GSL in DS-GSL form was eluted at 9.6 min and showed signals at m/z 415, 437 and 453, corresponding to [M + H]+, [M + Na]+ and [M + K]+, respectively, by an LC-ESI-MS analysis. The positive-ion ESI-MS/MS data indicated that the unknown DS-GSL had a molecular weight of 415 ([M + H]+) with two predominant fragments of m/z 253 ([M – C6H11O4 + H]+, 100%) and 164 ([M – C6H11O3 – C2H2NO2 + H]+, 25%). In addition, the molecular formula of the substance was determined to be

![Fig. 1. The Structure of 4-(Cystein-S-yl)butyl Desulfo-Glucosinolate](Image)
indicated a CH–CH coupling by a HH-COSY analysis. These signals and 2.84–2.95 ppm, and were correlated with geminal L-Ser (13C).

**Preparation of 4-(Cystein-S-yl)butyl Desulfo-Glucosinolate (DS-GSL) (1) with Dimeric 4-Mercaptobutyl DS-GSL (2) and Authentic t-Ser (5).**

3. 2-mercaptoethanol used for breaking the S–S bond of 2; 4. 4-mercaptobutyl DS-GSL, the cleaved product of 2.

C_{13}H_{28}O_{12}N_{2}S_{2}, based on its MALDI-TOF-MS chromatogram (ion at m/z 413.10291 [M – H]−). MALDI-TOF-MS provides a quick and simple method for GSL analysis because very little purification is required.24 From the 1H-NMR analysis of the unknown GSL in D_{2}O, the proton signals at δ 3.27–4.83 ppm and 1.55–2.46 ppm indicate typical signal patterns of a carbohydrate and 1,4-disubstituted butyl group, respectively.1,6,15,25 Other signals were observed at δ 3.72–3.75 and 2.84–2.95 ppm, and were correlated with geminal coupling by a HH-COSY analysis. These signals indicated a CH–CH_2 unit with an asymmetric carbon. Moreover, the ethyl group was estimated to be S-alkyl-cysteine, as the signal pattern was similar to that of aspartic acid and S-methyl-cysteine26,27 because the carbonyl signal was observed only once in the 13C-NMR spectrum. The 1H-signal at δ 2.47 ppm of one side end of a 1,4-disubstituted butyl group was correlated with the 13C-signal at δ 32.4 ppm of β-position of the cysteine group in the HMBC spectrum. The overall structure of the unknown DS-GSL was tentatively assigned as 4-(cystein-S-yl)butyl DS-GSL (Fig. 1). This is based on an analysis of the connections of individual partial structures by HMBC measurements and a molecular formula based on MALDI-TOF-MS data (calc. for C_{13}H_{25}O_{8}N_{2}S_{2}, 413.10469; found at m/z 413.10291 [M – H]−).

**Preparation of the unique DS-GSL**

To match the chemical structure with the putative formula, 4-(cystein-S-yl)butyl DS-GSL (1) was prepared by a base-catalyzed replacement reaction between dimeric 4-mercaptopbutyl DS-GSL (2), which had been isolated and purified from the rocket leaves,11 authentic t-Ser (5) and 2-mercaptoethanol (3, Fig. 2). After this replacement reaction, the solution was applied to LC-ESI-MS/MS, and several total ion chromatograms were obtained and characterized by the MS/MS data (data not shown). The compounds at retention times (t_{R}) 20.7, 2.9, 3.9 and 14.1 min of MS were 2 (ion at m/z 653, [M + H]^{+}), 5 (m/z 106 [M + H]^{+}), 1 (m/z 415, [M + H]^{+}) and 4 (m/z 328, [M + H]^{+}), respectively. The NMR spectra and the replacement reaction of the unique GSL suggest that its compositional formula is (C_{13}H_{25}O_{8}S–C(NO_{3})_{3}–(CH_{2})_{4}–S–CH_{2}–CH(NH_{2})–COOH) as a sulfate of 4-(cystein-S-yl)butyl DS-GSL (1), which consists of 4-mercaptopbutyl GSL and t-Ser. It is named “glucorucolamine” as a trivial name based on its ammonia sensitivity.

**Putative biosynthesis of the 4-(cystein-S-yl)butyl GSL**

The putative biosynthetic route to the 4-(cystein-S-yl)butyl GSL may begin with glutamine or glutamate induced by NH_{4}^{+} excess/toxicity according to the recycling of ammonia in plants (Fig. 3).28,29 When the glutamic acid and ATP supply are sufficient, NH_{3} generated in the assimilation of NH_{4}^{+} is incorporated into the carboxyl group of glutamate, and converted to a nontoxic form as the –NH_{2} group of glutamine.30 t-Ser is synthesized from hydroxypyruvate and t-alanine, an...
important function in the delivery of nitrogen from l-glutamate.\(^{31}\) When the l-Ser pool size is increased, 4-(cystein-S-yl)butyl GSL is synthesized by l-Ser and 4-mercaptopbutyl GSL. To our knowledge, this mechanism has not previously been described.

To provide further confirmation of the putative biosynthetic route, the free amino acids were analyzed in leaves hydroponically-grown in 25% and 100% NH\(_4\)\(^+\)-N nutrient solutions (Fig. 4).\(^{13}\) The excess NH\(_4\)\(^+\) (100% NH\(_4\)\(^+\)-N) had large stimulatory effects on l-Ser and the ammonium concentration, and glutamine, glutamic acid and cystine were slightly more in comparison with 25% NH\(_4\)\(^+\)-N growth, while alanine was less, presumably due to its quick turnover for the synthesis of l-Ser. These results are in good agreement with previous reports,\(^{32}\) in which this is illustrated by the observations that the accumulation of ammonium and glutamine during the day was accompanied by an accumulation of l-Ser and glycine. Details of the biosynthetic pathway to l-Ser. These results are in good agreement with previous reports,\(^{32}\) in which this is illustrated by the observations that the accumulation of ammonium and glutamine during the day was accompanied by an accumulation of l-Ser and glycine. Details of the biosynthetic pathway to 4-(cystein-S-yl)butyl GSL that was identified in this study are presently unknown. However, based on the results of the preparation and amino acid analysis, 4-(cystein-S-yl)butyl GSL would probably be synthesized from l-Ser with 4-mercaptopbutyl GSL, though its formation as a true in-plant metabolite is questionable.

\(^{4}\)-Mercaptobutyl isothiocyanate (HS–CH\(_2\)–CH\(_2\)–CH\(_2\)–N=S) and its precursor, together with their corresponding disulfide and dimeric GSLs, have previously been identified from the leaves of rocket salad.\(^{6,12}\) 4-Mercaptobutyl GSL (4) may be a key compound linking 4-(cystein-S-yl)butyl GSL (1), dimeric 4-mercaptopbutyl GSL (2) and \(4\)-\(\beta\)-d-glucopyranosyldisulfanyl)butyl GSL in the leaves of rocket salad.\(^{1,6,12}\) However, Bennett et al.\(^{5}\) have claimed that dimeric 4-mercaptobutyl GSL was an artifact formed from 4-mercaptopbutyl GSL by non-enzymatic oxidation during the extraction process. The DS-GSL analysis by boiling-MeOH extraction and the conversion of DS-GSL would readily cause the oxidation of 4-mercapto-

\begin{figure}[h]
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\includegraphics[width=\textwidth]{fig4.png}
\caption{Effect of Ammonium: Nitrate Percentage Molar Ratio on the Free Amino Acid Concentrations (mg 100 g\(^{-1}\) fresh wt.)\(^{a}\) in the Leaves of Rocket Salad.}
\footnotesize{\(^{a}\)Each value is the mean \pm standard deviation (\(n = 2\)). \(^{b}\)Percentage molar ratio of NH\(_4\)\(^+\) to NO\(_3\)\(^-\) at the same total nitrogen level (10 mmol l\(^{-1}\)): 25, 25% of NH\(_4\)\(^+\); 100, 100% of NH\(_4\)\(^+\).}
\end{figure}

butyl GSL, and it can thus be expected that not only would the dimeric 4-mercaptopbutyl GSL be present but also other disulfide GSLs corresponding to the oxidation (mixed disulfides) with any sulfur-containing metabolites such as thioglucose, glutathione and l-cysteine.\(^{33}\)

However, it is obvious that if the level of l-Ser is much higher in the plant grown in the 100% NH\(_4\)\(^+\)-N nutrient solution, it would mainly be used for the formation of 4-(cystein-S-yl)butyl GSL and two indolyl GSLs (glucobrassicin and 4-methoxyglucobrassicin) to reduce the NH\(_4\)\(^+\) excess.\(^{13}\) In addition, if the formation of 4-(cystein-S-yl)butyl GSL only results in the oxidation of 4-mercaptopbutyl GSL during the extraction process for rocket salad, it is not explainable why 4-mercaptopbutyl GSL was not conjugated with the free l-Ser during the analysis of DS-GSL when the plants were cultivated with a relatively low percentage of NH\(_4\)\(^+\)-N to NO\(_3\)\(^-\) by a combination of the NO\(_3\)\(^-\)-N and NH\(_4\)\(^+\)-N nutrient solutions.\(^{13,33}\) A ligase of the enzyme which synthesizes the 4-(cystein-S-yl)butyl GSL with l-Ser and 4-mercapto-

b butyl GSL would be activated by the 100% NH\(_4\)\(^+\)-N treatment, but even this pathway to 4-(cystein-S-yl)butyl GSL remains speculative.

In summary, 4-(cystein-S-yl)butyl GSL and the two indolyl GSLs of rocket salad were enhanced in a 100% NH\(_4\)\(^+\)-N nutrient solution culture. Such a high N application level, particularly with NH\(_4\)\(^+\)-N as the N source, would be likely tied to the two N in the molecular structures of these GSLs. Although the biosynthesis of 4-(cystein-S-yl)butyl GSL from applied NH\(_4\)\(^+\)-N remains to be elucidated, the evidence seems to suggest that there exists a second pathway from NH\(_4\)\(^+\)-N in the formation of GSLs, particularly of 4-(cystein-S-yl)butyl GSL.

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