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Determination of the absolute configuration of the glucosinolate methyl sulfoxide group reveals a stereospecific biosynthesis of the side chain

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ABSTRACT

Glucosinolates are plant metabolites containing an anionic nitrogeneous thioglucosidic core structure and a structurally diverse amino acid-derived side chain, which after hydrolysis by thioglucohydrolases (myrosinases) afford biological active degradation products such as nitriles and isothiocyanates. Structural diversity in glucosinolates is partially due to enzymatic modifications occurring on the preformed core structure, like the recently described oxidation of sulfides to sulfoxides catalyzed by a flavin monooxygenase identified in *Arabidopsis thaliana*. The enzyme product, 4-methylsulfinylbutylglucosinolate, bears a chiral sulfoxide group in its side chain. We have analyzed the epimeric purity of 4-methylsulfinylbutylglucosinolate by NMR methods using a chiral lanthanide shift reagent. The absolute configuration of the sulfoxide group has been established by comparing the ^{1}H NMR spectra of the two sulfoximine diastereomers of natural 4-methylsulfinylbutylglucosinolate. According to our data, 4-methylsulfinylbutylglucosinolate isolated from broccoli and *A. thaliana* is a pure epimer and its sulfoxide group has the R_S configuration. The product of the *A. thaliana* flavin monooxygenase has these same properties demonstrating that the enzyme is stereospecific and supporting its involvement in glucosinolate side chain formation.

1. Introduction

Glucosinolates are plant metabolites defined by the presence of a core structure, containing a β-D-thioglucopyranose residue and a *N*-hydroxyiminosulfate ester, which is linked to a variable side chain. More than a hundred glucosinolates have been described (Halkier and Gershenzon, 2006) and part of this structure diversity arises from secondary modifications carried out on the core structure. 4-Methylsulfinylbutylglucosinolate (glucoraphanin, **1**, Fig. 1) is an abundant glucosinolate in the commercially important crops of the genus *Brassica* (broccoli, cabbage) as well as in the Col-0 accession of the model plant *Arabidopsis thaliana* (Fahey et al., 2001) of the Brassicales order. The biosynthesis of aliphatic glucosinolates like **1** is conceptually divided in three phases starting from methionine; first, the amino acid chain is elongated; second, the core structure is formed; and third, the aliphatic chain undergoes secondary modifications (Halkier and Gershenzon, 2006). An example of a secondary modification is the conversion of **3** to **1** (Fig. 2), which involves the oxidation of the thiol residue to a sulfoxide group. Such sulfoxide groups are found in many glucosinolates (Fahey et al., 2001).

X-ray analyses have shown that sulfoxides have a trigonal pyramidal geometry (Carey et al., 1976; Kodama et al., 1976). Because their pyramidal inversion barrier typically is in the range of 35–43 kcal mol^{-1} (Bentley 2005), stable sulfoxide enantiomers are possible if the two substituents are different, as in **1**. However, the stereochemistry of the sulfoxide moiety of glucosinolates is rarely considered although its configuration may be critical for its biological activity and the biological activity of glucosinolate hydrolysis products formed by myrosinase catalysis following plant damage (Halkier and Gershenzon, 2006). In the case of **1**, its myrosinase-mediated hydrolysis product, sulforaphane (**2**, Fig. 1) has been intensively investigated for its anticancer activity, attributable to its ability to modulate phase I and phase II detoxification pathways (Fimognari and Hrelia, 2007). Nevertheless only one study has evaluated the influence of the stereochemistry of the sulfoxide group of **2** on its anticancer activity (Zhang and Tang, 2007).

Sulfur chirality has not been as widely addressed as carbon chirality in natural products and medicinal chemistry. Nevertheless, there are some illuminating examples that demonstrate the relevance of sulfoxide stereochemistry to biological activity. For

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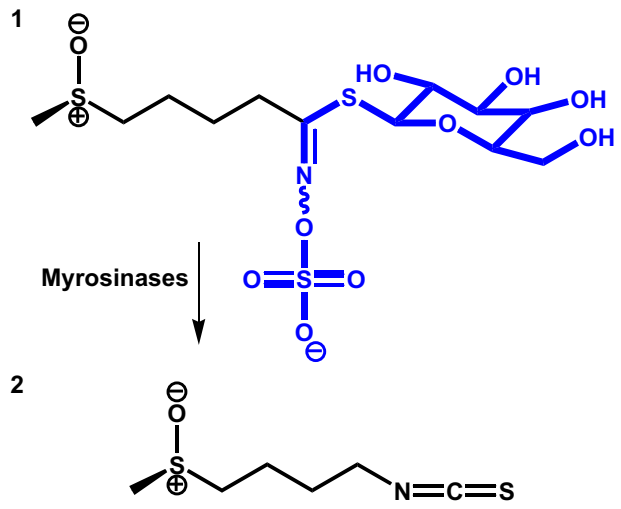

Fig. 1. *Hydrolysis of glucosinolates*. Plants of the Brassicaceae produce glucosinolates, such as 4-methylsulfinylbutylglucosinolate (**1**). After tissue damage the enzyme myrosinase hydrolyzes the glucosinolates to products including isothiocyanates, such as sulforaphane (**2**), which is a chiral molecule due to the presence of the asymmetric sulfoxide group. The core structure common to all glucosinolates is shown in **1** with bold (blue) bonds.

instance, S_CS_S- but not S_CR_S-methionine sulfoxide was recognized by the enzyme gamma-glutamyl transpeptidase, responsible for the transfer of a gamma-glutamyl moiety from a donor substrate to acceptors, such as amino acids and water (Lherbet and Keillor, 2004). In addition, the S_S enantiomers of hexyl methyl sulfoxide and methyl phenyl sulfoxide bound more tightly (about 5–8-fold) than the R_S enantiomers to horse liver alcohol dehydrogenase (Cho and Plapp, 1998). A drug called Nexium® (esomeprazole, that is the pure S_S isomer), is often prescribed instead of the racemic mixture, Prilosec® (Losec®, omeprazole), owing to its better clinical properties as a proton pump inhibitor (Olbe et al., 2003).

Knowledge about the epimeric purity and configuration of the glucosinolate side chain sulfoxide moiety would also shed light on the specificity of glucosinolate biosynthetic enzymes. The *S*-oxidation of methylthioalkyl glucosinolates leading to the formation of the sulfoxide in **1** is carried out by a flavin monooxygenase (FMO) encoded by the gene $FMO_{GS\text{-}OX1}$ (*At1g65860*) recently identified in *A. thaliana* using a combination of *in silico* and biochemical data (Hansen et al., 2007). But, it is not known if this enzyme produces a chiral sulfoxide, and if so which configuration.

The enantiomeric purity of some sulfoxides has been determined using chiroptical methods or chiral chromatography, while their absolute configuration has been determined via X-ray crystallography, nuclear magnetic resonance (NMR) spectroscopy and chiroptical methods like optical rotatory dispersion (ORD) and circular dichroism (CD) (Donnoli et al., 2006). To date no study has reported on the epimeric purity or the absolute configuration of sulfoxides in intact glucosinolates. However, the combination of ORD and X-ray data showed that the glucosinolate hydrolysis product **2** isolated from several plant sources is an optically active compound with the R_S absolute configuration (Mislow et al., 1964; Cheung et al., 1965), but nothing is known about sulfoxide-containing glucosinolates nor about the stereospecificity of the sulfoxides produced by the flavin monooxygenase enzyme (*At1g65860*).

In this investigation, we set out to develop an accurate, sensitive method to determine the stereochemistry of the sulfoxide group of glucosinolate side chains that could be applied to plant metabolites or the products of an enzyme assay. Chiral HPLC and chiroptical methods tested failed to determine the epimeric purity of glucosinolates at their sulfoxides, most probably because additional stereocenters also interacted with the stationary phase or the absorption spectrum was dominated by other functional groups in the molecule, respectively. Hence we turned to NMR using a chiral lanthanide shift reagent (CLSR) which relies on the anisotropy generated by europium to resolve signals for epimers. The absolute configuration of the sulfoxide in glucosinolate side chains was assessed via an extension of the Mosher method by preparing the *N*-(methoxyphenylacetyl)sulfoximine derivative according to Yabuuchi and Kusumi (1999). These methods were developed for **1** extracted from *Brassica oleracea* var. *italica* (broccoli) which was available in a large amount and then applied to **1** isolated from *A. thaliana* and **1** produced by the *At1g65860* flavin monooxygenase in of glucosinolate side chain biosynthesis *A. thaliana*. The three different samples of **1** were all shown to be epimerically pure and to possess the same stereochemistry.

2. Results

To determine the epimeric purity of **1** with respect to the sulfoxide group of its side chain, semi-synthetic **1** (R_SS_S-**1**, Fig. 2) was prepared as a reference by H_2O_2 oxidation of 4-methylthiobutylglucosinolate (**3**), which was isolated from *Eruca sativa* seeds (Fig. 2). Chiral chromatography and chiroptical methods failed to resolve the epimers. Chromatograms of semi-synthetic **1** after separation on a chiral column showed only a single compound, whose CD spectrum was indistinguishable from the spectrum of **1** isolated from broccoli, showing a plain curve centered near 230 nm. This absorption band was most probably due to the transitions of the chromophore belonging to the core glucosinolate structure, because it was also present in the CD spectrum of allyl glucosinolate, which does not contain a sulfoxide group. Turning to ^{1}H NMR, when **1** from *B. oleracea* and semi-synthetic **1** (racemic at the sulfoxide group) were analyzed in the presence of a chiral lanthanide shift reagent (CLSR), their spectra differed. In the ^{1}H NMR spectra of semi-synthetic **1**, the signal at δ 2.83 corresponding to the methyl-sulfoxide group was shown to consist of two non-equivalent signals (Fig. 2a) an HSQC experiment confirmed the phenomenon of non-equivalence demonstrating that the two sulfoxide ^{1}H signals were each correlated to a different ^{13}C signal with a different chemical shift (data not shown). In contrast, when **1** isolated from *B. oleracea* was analyzed using the same ^{1}H NMR conditions, the methyl sulfoxide signal at δ 2.85 did not show non-equivalence (Fig. 2b). Thus, **1** from *B. oleracea* was likely to be a pure epimer at the sulfoxide group. To confirm this epimeric purity determination, a sample was prepared mixing equimolar amounts of **1** from *B. oleracea* and semi-synthetic **1**. The ^{1}H NMR spectrum (Fig. 2c) showed an increase in the magnitude only for the downfield signal of the methyl sulfoxide and integration of the two signals gave a 3:1 ratio.

To determine the absolute configuration of the methyl sulfoxide group of **1** isolated from *B. oleracea*, the R_C- and S_C-*N*-(methoxyphenylacetyl)sulfoximine (*S*–*N*–MPA) derivatives (**4**, Fig. 3) were prepared (Yabuuchi and Kusumi, 1999). This method, in analogy to the Mosher method, takes advantage of chemical shift differences ($\Delta\delta = \delta S_C - \delta R_C$) between corresponding signals in the spectra of the diastereomeric R_C and S_C sulfoximine derivatives. Many chiral sulfoxides have been studied following this approach and a clear correlation between absolute configuration and ^{1}H NMR shift differences has been observed (Kusumi et al., 2006) when $CDCl_3$ was used as a NMR solvent. Unfortunately, the *S*–*N*–MPA derivatives of 4-methylsulfinylbutylglucosinolate (**4**) are insoluble in unpolar solvents such as $CDCl_3$ and C_6D_6, which are suitable for the elucidation of absolute configuration (Kusumi et al., 2006). Thus, we hydrolyzed **4** using myrosinase to produce an *S*–*N*–MPA derivative of sulforaphane (**5**, Fig. 3). The ^{1}H NMR signals in C_6D_6

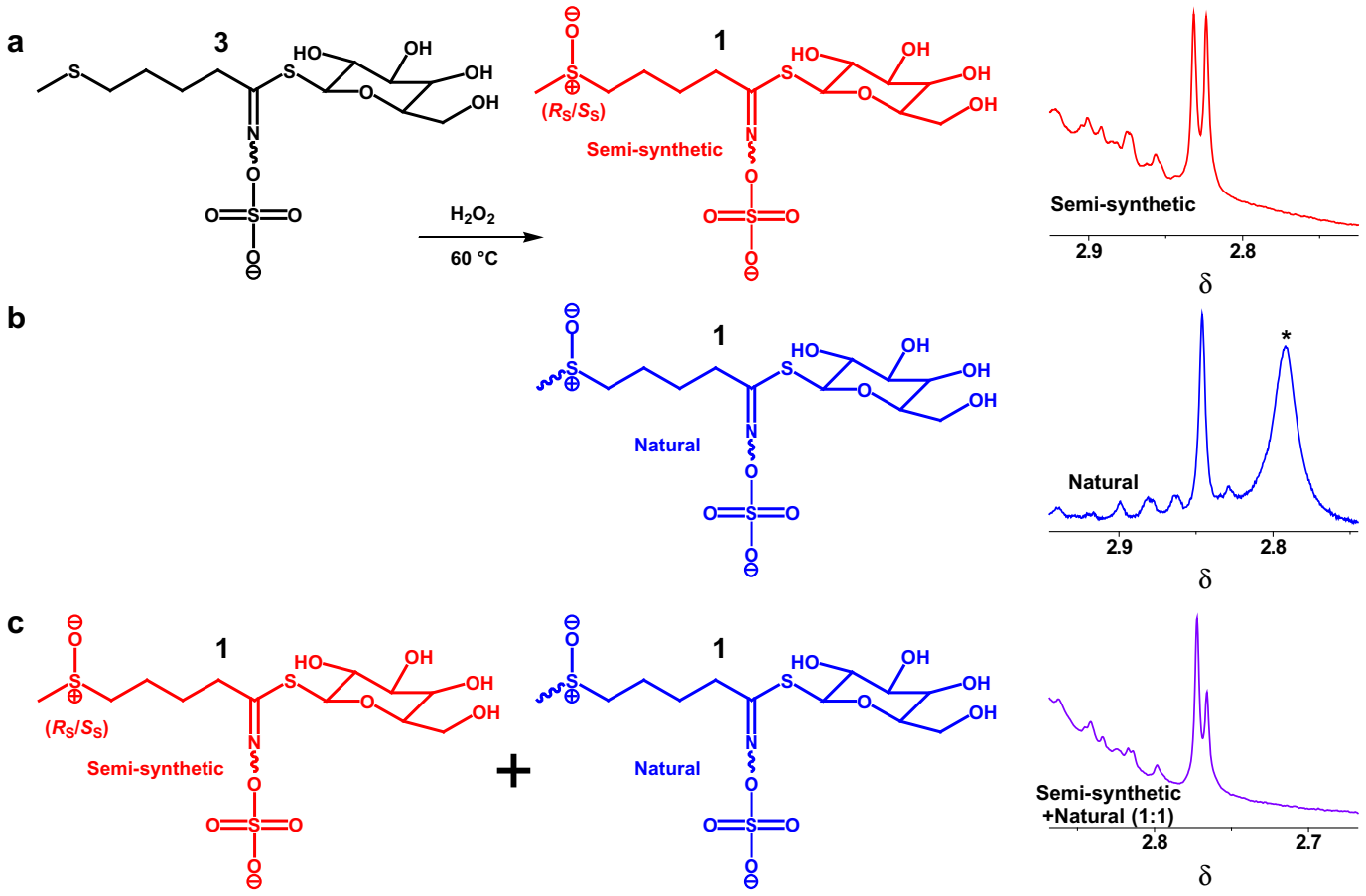

Fig. 2. *Epimeric purity determination of 4-methylsulfinylbutylglucosinolate* (**1**) *from* Brassica oleracea *var.* italica *(broccoli) in relation to its sulfoxide group*. Compound **1** racemic with respect to its sulfoxide group [(1-R_S):(1-S_S), 50:50] was produced by symmetric oxidation of glucoerucin (**3**) with H_2O_2 for comparison of its methyl sulfoxide ^{1}H NMR signal (a) with that of **1** isolated from broccoli (b) in the presence of a chiral lanthanide shift reagent (CLSR). The signal at δ 2.8 in b (∗) corresponds to HDO. To confirm the apparent epimeric purity of **1**, an equimolar mixture of semi-synthetic R_SS_S-**1** and **1** from broccoli was prepared. The resulting ^{1}H NMR spectrum (c) showed enhancement of the downfield signal only (ratio between signals = 3:1), indicating that **1** from broccoli is a pure epimer at the sulfoxide. The *x* axis has been shifted slightly to make the signals easier to compare. Slight differences in the chemical shift of the methyl sulfoxide protons are due to differences in the relative water contents of the samples. Spectra were recorded at 400 MHz in CD_3CN/CD_3OD (9:1) at 50 °C.

of the methyl and four methylene groups of **5** showed chemical shift differences between the R_C and the S_C diastereoisomers. Unambiguous signal assignment for the two *S*–*N*–MPA diastereomers of **5** was carried out based on ^{1}H–^{1}H COSY and HSQC experiments. $\Delta\delta$ values for the methylene signals **I**, **II** and the methyl signal **V** were directly calculated from experimental ^{1}H NMR measurement, whereas $\Delta\delta$ for the methylene signals **III** and **IV** were calculated using δ values obtained from a computer simulation of the eight-spin system. Simulated δ values were as follows: **5**-S_C: **I** 2.27, **II** 0.63, **III** 1.02, **III′** 0.94, **IV** 2.52 and **IV′** 2.29 and for **5**-R_C: **I** 2.24, **II** 0.58, **III** 0.93, **IV** 2.50 and **IV′** 2.19. The chemical shift differences between the R_c and S_c sulfoximine derivatives of **5** are depicted at the bottom of Fig. 3 with the diameters of circles for each ^{1}H NMR signal proportional to $\Delta\delta$ magnitude and colors representing the sign of $\Delta\delta$ (Fig. 3). The sign of $\Delta\delta$ is the parameter that provides information about the configuration of the sulfoxide, because it is the result of the space-oriented aromatic shielding effect produced by the phenyl ring in MPA which selectively shields the methyl group (**V**) in the S_C configuration or the methylene protons (**I**–**IV**) in the R_C configuration. In the sulfoximine model, positive and negative $\Delta\delta$ lie on the left and right hand of the *N*–*S*–*O* plane, respectively (Kusumi et al., 2006). Because $\Delta\delta$ is negative for the methyl group and positive for the methylene protons in **5**, the absolute configuration of the sulfoxide in **5**, and by extension in **4** and **1** from *B. oleracea*, is inferred to be R_S. ^{1}H NMR analyses were also performed on **1** extracted from *A. thaliana* rosette leaves and **1** produced by the *A. thaliana* flavin monooxygenase expressed in *Escherichia coli* spheroplasts. In both cases, **1** was enantiomerically pure at the methyl sulfoxide position and had an R_S absolute configuration, as evidenced by the presence of only one signal without CLSR. In the presence of CLSR, there were non-equivalent signals in the spectra with increase only in the downfield signal (Fig. 4).

3. Discussion

The asymmetric sulfoxide group of glucosinolate side chains creates a chiral center at an atom other than carbon due to its stable trigonal pyramidal geometry. Glucosinolate **1** isolated from *B. oleracea* var. *italica* and *A. thaliana* was found to be epimerically pure with an R_S absolute configuration. This result is consistent with measurements of glucosinolate-derived isothiocyanates made by ORD over 40 years ago (Cheung et al., 1965). The product **1** of the *A. thaliana* flavin monooxygenase enzyme $FMO_{GS\text{-}OX1}$, reported to oxidize the methylsulfide group of 4-methylthiobutylglucosinolate (**3**) to **1** has the same properties. These data indicate that the enzyme is stereospecific and support its involvement in glucosinolate side chain biosynthesis. Related enzymes may catalyze *S*-oxidation of methylthioalkyl glucosinolates throughout the Brassicaceae since a variety of different sulfoxide bearing

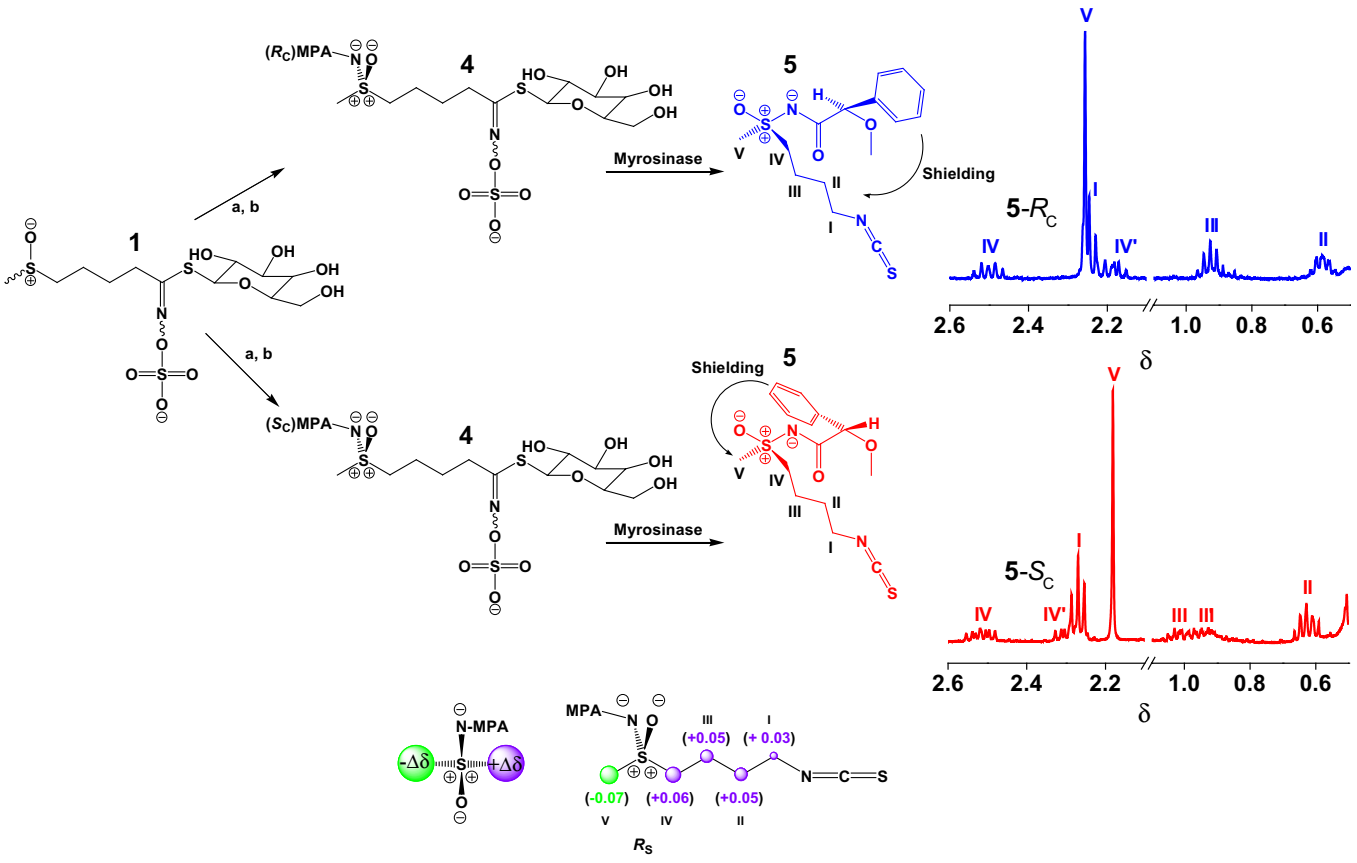

Fig. 3. *Absolute configuration determination of the sulfoxide in 4-methylsulfinylbutylglucosinolate* (**1**) *by NMR using the sulfoximine method*. For solubility reasons, myrosinase *S*–*N*–MPA derivatives **5** were used instead of the 4-methylsulfinylbutylglucosinolate *S*–*N*–MPA products **4** for ^{1}H NMR analyses in C_6D_6. Comparison of δ values between the S_C and R_C diastereoisomers of *S*–*N*–MPA derivatives **5** resulted in chemical shift differences ($\Delta\delta = \delta S_C - \delta R_C$). The sign of $\Delta\delta$ was compared with the sulfoximine model at the bottom of the figure (Kusumi, 2006) and from these data the absolute configuration of the sulfoxide in **5**, and by inference in **1** and **2**, was determined as R_S. (a) MSH and (b) Coupling reagent; MPA: Methoxyphenylacetyl group. Structures of **5** are represented in an idealized perspective. Spectra were recorded at 25 °C.

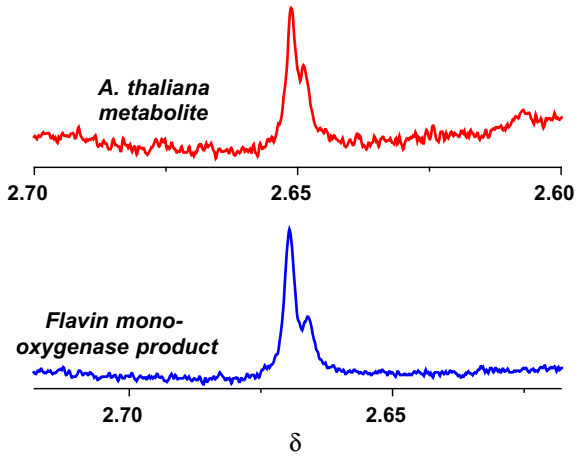

Fig. 4. *Epimeric purity determination of 4-methylsulfinylbutylglucosinolate* (**1**) *isolated from* A. thaliana *and produced by* A. thaliana *flavin monooxygenase in relation to its sulfoxide group*. ^{1}H NMR spectra of equimolar mixtures of synthetic R_SS_S-**1** and **1** from *A. thaliana* showed enhancement of the downfield signal exclusively, as with **1** extracted from broccoli. Therefore, **1** is in both cases enantiomerically pure with an R_S absolute configuration. The *x*-axis has been shifted slightly to make the signals easier to compare. Slight differences in the chemical shift of the sulfoxide protons are due to differences in the relative water contents of the samples. Spectra were recorded at 50 °C.

isothiocyanates measured display levorotatory ORD spectra (Cheung et al., 1965), suggesting that the diverse sulfoxide bearing glucosinolate have the same R_S stereochemistry. The NMR methods used here for determining epimeric purity and absolute configuration have an advantage over the chiroptical spectroscopy previously used in that they can be applied directly on glucosinolates in a simple, reliable manner. Whether the absolute configuration of the side chain sulfoxide in glucosinolates or their hydrolysis products is important for biological activity in anticancer or plant-herbivore interactions, is still an open question. The methods developed in the present work should help to shed light on these topics and guide efforts to produce medically or agriculturally valuable glucosinolates by biotechnological or pure synthetic methods.

The fact that **1** is enantiomerically pure may shed some light on the subcellular localization of glucosinolates in plants since sulfides are easily converted into sulfoxides under oxidative conditions. Spontaneous oxidation of 4-methylthiobutylglucosinolate (**3**) would lead to the production of racemic **1** with respect to the sulfoxide group (R_SS_S-**1**). The enantiomeric purity of the isolated **1** in this study suggests localization in a compartment in which oxidative processes are carefully controlled. Glucosinolates have been hypothesized to be stored in vacuoles (Grob and Matile, 1979; Helmlinger et al., 1983). Interestingly, the oxidation-sensitive **3** is much more abundant in the seeds of most *A. thaliana* accessions than in the foliage (Brown et al., 2003). Another possibility might be that some spontaneous oxidation of **3** does occur *in vivo*, but that this is followed by substrate-specific reduction of S_S-**1** back to **3**, yielding a residual pure epimer, R_S-**1**. Stereospecific enzymatic reduction of methionine sulfoxide residues on

proteins is known to be performed by sulfoxide reductases (Boschi-Muller et al., 2008) and enzymes catalyzing specific reductions of one sulfoxide configuration or the other have been identified in *A. thaliana* (Rouhier et al., 2006). Whether these enzymes can reduce glucosinolate side chains or there is instead a specific-sulfoxide reductase for conversion of **1** to **3** is not known.

4. Experimental

4.1. Extraction of glucosinolates

The glucosinolate, 4-Methylsulfinylbutylglucosinolate (glucoraphanin, **1**), was isolated from broccoli seeds (*Brassica oleracea* L.; Juliwa Gemüse, Heidelberg, Germany), *Arabidopsis thaliana* Col-0 leaves grown in greenhouse facilities and *E. coli* spheroplast culture. 4-Methylthiobutylglucosinolate (glucoerucin, **3**) was isolated from *Eruca sativa* L. seeds (Saatzucht Quedlinburg, Germany) as described previously (Thies, 1988) with a final purification by HPLC (see method 1). Their structures were confirmed by electrospray LC–MS (method 1) using diagnostic ions detected in MS/MS mode (*m*/*z* 436.0 [$M^{\ominus}$], 372, 259 for **1** and *m*/*z* 420.0 [$M^{\ominus}$], 340, 259, for **3**).

4.2. Synthesis of R_SS_S-4-methylsulfinylbutylglucosinolate (R_SS_S-**1**)

4-Methylthiobutylglucosinolate (**3**) (3 mg) was dissolved in H_2O (0.8 ml), H_2O_2 (0.03 ml) was added, and the mixture was heated at 60 °C for 30 min with agitation to afford **1** (Iori et al., 1999) which was then purified by preparative HPLC (method 1). The structure was confirmed by LC–MS using diagnostic ions in MS/MS mode (*m*/*z* 436.0 [$M^{\ominus}$], 372, 259).

4.3. Synthesis of S_C- and R_C-*N*-(methoxyphenylacetyl)sulfoximine derivatives (**4**) of -4-methylsulfinylbutylglucosinolate

Synthesis of the sulfoximine derivatives of **1** isolated from broccoli followed the one-pot method described by Yabuuchi and Kusumi (1999), with the following modifications: In the first step, DMF was used instead of CH_2Cl_2 as the solvent and *O*-mesitylsulfonylhydroxylamine (MSH) was produced as described by Krause (1972). The second reaction (condensation with methoxyphenylacetic acid) was carried out without modifications of the original procedure and the compound was purified by preparative HPLC (method 1). The structure of the products was confirmed by electrospray LC–MS (method 1) with diagnostic ions in MS/MS mode (*m*/*z* 599.0 [$M^{\ominus}$], 436, 372, 259).

4.4. Enzymatic hydrolysis of *N*-(methoxyphenylacetyl)sulfoximine derivative **4**–**5**

The S_C R_C sulfoxime derivatives **4** from broccoli were each dissolved in 500 μl of 100 mM Tricine (pH 8.0). Myrosinase (2 mg) (Sigma, Germany) was added and the mixture stirred for 30 min at 25 °C. The structure of the *S*–*N*–MPA derivatives **5** obtained were confirmed by electrospray LC–MS (method 2) with diagnostic ions in MS/MS mode (*m*/*z* 341.0 $[M+H]^{\oplus}$, 309, 281) and the products were purified using HPLC (method 2).

4.5. Assay of A. thaliana flavin monooxygenase

An expression construct containing the open reading frame of the $FMO_{GS\text{-}OX1}$ line cloned into a pBAD–TOPO vector was cloned into *E. coli* and expressed as described (Hansen et al., 2007). An overnight culture (1 ml) started from single cell colony in LB (with 100 μg ml^{-1} ampicillin) was used to inoculate 100 ml of LB medium supplemented with 50 μg ml^{-1} carbicillin and the culture was grown at 37 °C to OD_{600} = 0.5, at which time arabinose (final conc. 0.02%) was added. The culture was grown at 28 °C for 16 h and chilled on ice.

To isolate spheroplasts, the cells were centrifuged at 2500 *g* for 10 min at 4 °C, and the pellet resuspended in 8.3 ml 0.2 M Tris/HCl (pH 7.6) using a soft brush. Then, 1.42 g sucrose, 16.7 μl 0.5 M EDTA, 41.7 μl PMSF (0.1 M), 33.3 μl lysozyme (50 mg ml^{-1}), and 8.3 ml ice-cold water were added sequentially while slowly stirring. After an additional 30 min stirring at 4 °C, 166 μl 1 M cold MgOAc were added and the preparation centrifuged at 3000 *g* for 10 min at 4 °C, and resuspended in 1.8 ml 10 mM Tris (pH 7.6) with 14 mM MgOAc and 60 mM KOAc. The suspension was homogenized in a pre-cooled Potter–Elvehjem glass homogenizer. Then 5 μl RNase (10 mg ml^{-1}) and 10 μl DNase (5 mg ml^{-1}), were added and the mixture stirred for 30 min at 4 °C. After addition of 235 μl 87% glycerol (to a total of 30%), the spheroplasts were stored at −80 °C.

The assay was scaled up from that previously described (Hansen et al., 2007) to maximize product formation. An 0.5 ml volume of the spheroplast suspension was adjusted to 0.1 M Tricine buffer, pH 7.9, with 0.25 mM NADPH and 100 mM 4-metylthiobutylglucosinolate in a final volume of 1 ml. After an overnight incubation at room temperature, the products were extracted with 1 ml methanol and separated with HPLC method 1, as described below.

4.6. Circular dichroism

CD spectra were measured on a J-180 spectrometer (Jasco, Gross-Umstadt, Germany) with a 2 mm QS cuvette using MeOH as solvent. Spectra were recorded at 200−400 nm. Allyl glucosinolate used as a standard was purchased from Sigma.

4.7. High pressure liquid chromatography

All chromatographic analyses were carried out on HPLC-1100 series equipment (Agilent Technologies, Böblingen, Germany) using different conditions. *Method 1*: Chromatograph was coupled to an Esquire 6000 ESI-Ion trap mass spectrometer (Bruker Daltonics, Hamburg, Germany) operated in negative mode in the range *m*/*z* 50−1000. Skimmer voltage, −40 eV; capillary exit voltage, −146.7 eV; capillary voltage, 4000 V; nebulizer pressure, 35 psi; drying gas, 11 l min^{-1}; gas temperature, 330 °C. Elution was accomplished using an EC 250/4.6 Nucleodur Sphinx RP column (25 cm × 4.6 mm, 5 μm, Macherey-Nagel, Düren, Germany) coupled to a Supelguard™ LC-18 precolumn (2 cm × 2.1 mm, Supelco, Taufkirchen, Germany) with a gradient of 0.2% (v/v) formic acid (solvent A) and acetonitrile (solvent B) at a flow rate of 0.8 ml min^{-1} at 25 °C as follows: 0−100% (v/v) B (15 min), 100% B (3 min), 100−0% (v/v) B (6 s), 0% B (8 min 54 s). Eluent coming from the column was diverted in a ratio 4:1 before reaching the electrospray ionization unit. *Method 2*: Mass spectrometer operated in positive mode in the range *m*/*z* 30−500. Skimmer voltage, 40 eV; capillary exit voltage, 104.3 eV; capillary voltage, −4000 V; nebulizer pressure, 35 psi; drying gas, 11 l min^{-1}; gas temperature, 300 °C. Elution: Supelcosil™ LC-18 column (15 cm × 2.1 mm, 5 μm, Supelco) coupled to a Supelguard™ LC-18 precolumn (2 cm × 2.1 mm, Supelco) with a gradient of 0.2% (v/v) formic acid (solvent A) and acetonitrile (solvent B) at a flow rate of 0.25 ml min^{-1} at 25 °C as follows: 10−60% (v/v) B (15 min), 60−100% (v/v) B (6 s), 100% B (1 min 54 s), 100−10% (v/v) B (7 min 54 s). For isolation of compounds for NMR analysis, the same chromatographic conditions described in methods 1 and 2 were used but the column was connected to a fraction collector. *Method 3*: Chromatograph was connected to a UV detector operated at 230 nm. Elution was accomplished using a Chiralcel OD-H column (25 cm × 4.6 mm, 5 μm, Daicel) with methanol (isocratic) at a flow rate of 1 ml min^{-1} at 25 °C for 20 min.

4.8. Nuclear magnetic resonance spectroscopy

^{1}H NMR spectra were measured on an Avance 400 NMR spectrometer (Bruker Biospin, Rheinstetten, Germany) using a 5 mm BBI probe (^{1}H frequency 400.13 MHz) and on an Avance 500 NMR spectrometer (Bruker Biospin) equipped with a 5 mm TCI CryoProbe™ (^{1}H frequency 500.13 MHz). Chemical shifts are given as δ values using TMS as a reference (internal standard). Tris[3-(heptafluoropropylhydroxymethylene)-*d*-camphorate]europium(III) ($Eu(hfc)_3$) was used as the chiral lanthanide shift reagent (CLSR) (Alfa, Germany). $CDCl_3$, CD_3CN, and CD_3OD were purchased from Deutero GmbH (Kastellaun, Germany) and C_6D_6 from Euriso-top GmbH (Saarbrücken, Germany). Spectra with the CLSR were recorded at 400 MHz in CD_3CN/CD_3OD (9:1) using a 1:5 molar ratio (**1**:CLSR) at 50 °C. Computer simulations of spectra were run with the ACD/C + ^{1}H NMR predictor software (ACD/Labs, Canada).

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