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Cysteine Sulfoxides and Volatile Sulfur Compounds from *Allium tripedale*

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Allium tripedale Trautv. belonging to the subgenus *Nectaroscordum* grows naturally in the mountainous areas of northwest Iran. Leaves have a very strong and somewhat unpleasant taste and are widely used by the local population as a spicy vegetable. Three new cysteine based compounds were identified in *A. tripedale* as the first examples of sulfur containing 1-butenyl derivatives in nature. The compounds have been described as *o*-phthaldialdehyde (OPA) derivatives and identified as (+)-*S*-(1-butenyl)-L-cysteine sulfoxide (homoisoalliin) and its γ-glutamyl derivative as well as the γ-glutamyl derivative of *S*-(1-butenyl)-L-cysteine (desoxyhomoisoalliin). These cysteine sulfoxides have been also found in *Allium siculum* Ucria. As volatile compounds, di-(1-butenyl)-disulfide and the cepaene-like compounds di-(1-*S*-sulfoxymethyl-butyl)-disulfide, 1-*S*-sulfoxymethyl-butyl-1′-*S*-sulfoxy-1-butenyl-butyl-disulfide and 1-*S*-sulfoxymethyl-butyl-1′-*S*-sulfoxybutyl-butyl-disulfide could be tentatively identified by various MS experiments. Primary products resulting from the alliinase reaction of homoisoalliin seem to be highly unstable and were rapidly converted to the volatile compounds listed above.

KEYWORDS: ***Allium tripedale*; *Allium siculum*; cysteine sulfoxides; 1-butenyl-sulfur compounds; alliinase reaction**

INTRODUCTION

Many important crop plants belong to the genus *Allium* L., which contains more than 700 species worldwide. They occur mainly in the semiarid regions of Europe, North America, North Africa and Asia. Species like garlic (*A. sativum* L.) and onion (*A. cepa* L.) are commonly used since ancient times as a spice and vegetable but also as a medicinal plant. The beneficial antibiotic (*1*) and antidiabetic (*2*) activities of garlic are well-known and related to a great number of bioactive sulfur-containing compounds (*3*, *4*). Despite its long history in traditional medicine, especially in Asia, some *Allium* species are almost unknown and not well characterized, mostly due to geopolitical reasons. A main center of distribution is located in the countries Uzbekistan, Tajikistan and Afghanistan.

Among these, species of the subgenus *Nectaroscordum* (Lindl.) Asch. et Graebn., formerly known as separate genus *Nectaroscordum*, were only known in the Western World as ornamental plants because of their decorative inflorescences, which are rather different from usual onion umbels (**Figure 1b**). However, leaves from wild growing plants in the mountainous areas of northwest Iran and neighboring areas were collected in springtime and sold on local markets in huge amounts (**Figure 1a**,**c**). The fresh leaves, which were sometimes transported over hundreds of kilometers inside Iran, are highly prized by the local populations as a spice vegetable and were mostly used for the preparation of a special bread. If plant material of *A. tripetale* Trautv. is crunched, a very strong and somewhat unpleasant smell occurs immediately, which is accompanied by a slight eye irritation. The taste of *A. tripedale* is very pungent. Especially the irritation of eyes led to the assumption that the sulfur chemistry of this plant is related to that of common onion.

In a closely related species, *A. siculum* Ucria, *S*-butylcysteine sulfoxide (butiin, **5**, **Figure 2**) has been already reported (*5*). It was assumed that similar compounds also exist in *A. tripedale*. Besides this compound, *S*-methylcysteine sulfoxide (methiin, **1**, **Figure 2**), *S*-propylcysteine sulfoxide (propiin, **2**, **Figure 2**), *S*-allylcysteine sulfoxide (alliin, **3**, **Figure 2**) and *S*-(1-propenyl)cysteine sulfoxide (isoalliin, **4**, **Figure 2**) and in trace amounts *S*-ethylcysteine sulfoxide (ethiin) have been frequently reported for *Allium* species (*6*−*8*). Methiin is ubiquitous in the genus *Allium*. Alliin is the typical cysteine sulfoxide of garlic, whereas isoalliin is characteristic for common onion (*A. cepa*) and related species. These compounds have been also reported as their corresponding γ-glutamyl dipeptides. It was observed that, during storage of bulbs of *A. cepa*, the amount of γ-glutamyl cysteine sulfoxides decreased and the concentration of free cysteine sulfoxides increased by the same amount. Additionally, desoxy-derivatives of γ-glutamyl cysteine sulfoxides as precursors of sulfoxides have been observed in the genus *Allium*. It is proposed that, during the biogenetic pathway, cysteine sulfoxides can be stereoselectively oxidized only in the form of the corresponding γ-glutamyl dipeptides (*9*).

On the other hand, cysteine sulfoxides are subjected to the enzyme alliinase if plant material is crunched. Primary products of this reaction are thiosulfinates like allicin. In the case of common onion, the so-called "lachrymatory factor" (thiopropanal *S*-oxide) is formed. All these products are highly

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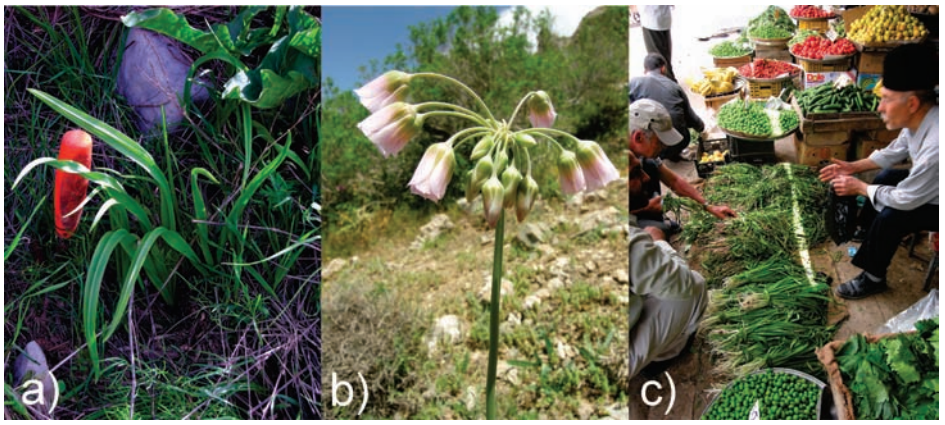

Figure 1. (**a**) Leaves of *A. tripedale* growing in the Razvand massif near Arak, Iran (Acc. No. 1157). (**b**) Inflorescence of *A. tripedale* found at the Rimeleh massif near Ghaleh Moradi (Acc. No. 1186). (**c**) Leaves of *A. jesdianum* (upper middle part of picture) and *A. tripedale* (lower middle part of picture) on the market of Khorramabad, sometimes with bulbs (Acc. No. 1173).

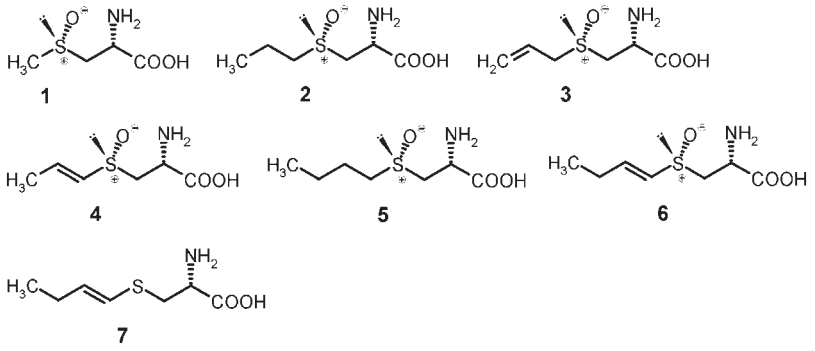

Figure 2. Chemical structures of already described cysteine sulfoxides (**1**−**5**) and cysteine sulfoxides as well as derivatives described for the first time in *A. tripedale* (**6**, **7**): **1**, methiin; **2**, propiin; **3**, alliin; **4**, isoalliin; **5**, butiin; **6**, homoisoalliin; **7**, desoxyhomoisoalliin (could be isolated as the corresponding γ-glutamyl derivative).

unstable and react to a huge variety of different sulfur compounds. In aqueous solutions, typical products are di- and trisulfides. A good overview of this rather complex chemistry was given by Block (*3*).

Based on the observations made in Iran it can be assumed that *A. tripedale* contains unique sulfur compounds, which might be different from those reported for *A. cepa* and *A. sativum*. Therefore, the aim of this study is the structure elucidation of sulfur compounds of this species, primarily structure elucidation of cysteine sulfoxides. Further on *A. siculum* was included in investigations, because it also belongs to the same subgenus *Nectaroscordum*. In contrast to *A. tripedale*, *A. siculum* is not a food plant, but widely used for ornamental reasons.

MATERIALS AND METHODS

Chemicals. Chemicals were purchased either from Merck (Darmstadt, Germany), Fluka (Deisenhofen, Germany) or Sigma (Munich, Germany). Millipore-grade water was used for all experiments.

Derivatization Reagent (OPA Reagent). For derivatization of amino acid derivatives, 140 mg of *o*-phthaldialdehyde was solubilized in 5 mL of methanol under magnetic stirring. Then, 200 μL of *tert*-butylthiol was added. Afterward, 50 mL of borate-buffer pH 9.5 was slowly mixed with the obtained solution. The reagent was stored for at least 12 h under light protection before usage.

Plant Material. The plant material (*A. nectaroscordum*, **Figure 1**) was collected in Iran in 2007. Voucher specimens have been deposited in the living plant collection at IPK Gatersleben, Germany, and at the living plant collection of the Plant Pests and Diseases Research Institute, Tehran, Iran. Additionally, sample material was obtained from the living plant collection of IPK Gatersleben, Germany (TAX No. 6082, collected by Dr. R. M. Fritsch in Armenia). The latter material was mainly used for preparative work. *A. siculum* was also obtained from the IPK living plant collection. The material was kindly provided by Dr. R. M. Fritsch. Due to the instability of leaf material (rapid discoloration), only bulbs were examined.

Extraction and Derivatization Procedure for Quantitative Determination of Amino Acid Derivatives. Bulbs were cleaned and sliced into pieces of about 500 mg and exactly weighted. These pieces were instantly placed in 20 mL of methanol and heated at 65 °C under reflux in order to inhibit enzymatic activity. After 10 min the pieces were homogenized and extracted with a mixture of methanol/water (1:1 v/v) by heating under reflux for 10 min. The extract was cooled and filtered, and the filter was washed carefully with methanol/water. The filtrate was evaporated under reduced pressure and stored at −20 °C before further use. For measurements, the extract was dissolved in 4.93 mL of OPA reagent, and 50 μL of *tert*-butylthiol was added. After reaction for 30 min in darkness at room temperature, 20 μL of iodoacetamide (1 M) was added. For quantitative measurements, 200 μL of the sample was diluted with 800 μL of water to give a final volume of 1.00 mL. For qualitative measurements, 500 μL of the sample was diluted in 500 μL of water. Out of this, 20 μL was injected into the HPLC device. A Merck Hitachi 7000 series HPLC system equipped with a VP 250/4 Nucleodur 100-5 C18 EC column (Macherey Nagel, Düren, Germany) was used for quantitative analysis. The column oven was set to 30 °C, and the UV detector was set to 334 nm. Calibration of the system was done with L-(+)-alliin standard synthesized according to Koch (*11*). The HPLC conditions were as follows: a 50 mmol phosphate buffer pH 6.5/acetonitrile gradient with a constant flow rate of 1 mL was used as mobile phase (A, 50 mmol phosphate buffer pH 6.5; B, acetonitrile). Gradient: 78% A for 20 min; 78−75% A over 29 min; 75% A for 1 min; 75−71% A over 4 min; 71% A for 1 min; 71−68% A over 8 min; 68−63% A over 2 min; 63% A for 10 min; 78% A for 10 min. Additionally, HPLC ESI-MS/MS measurements were performed on

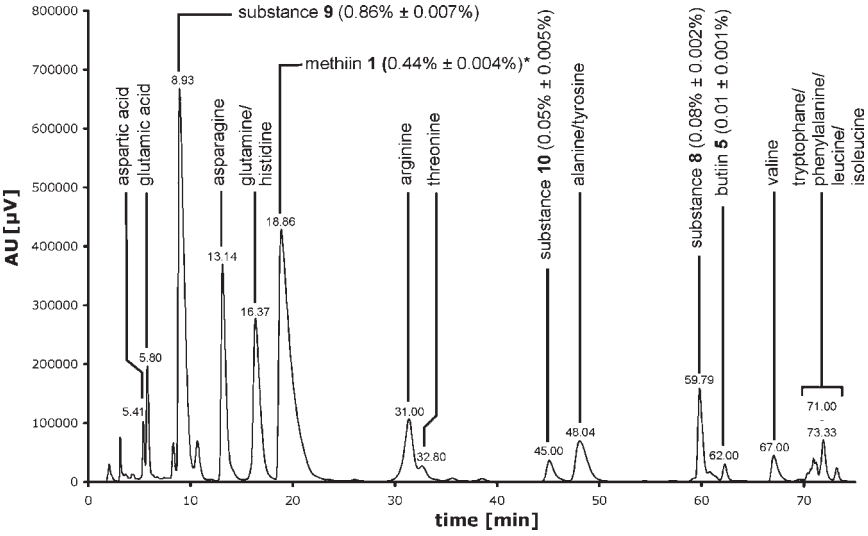

Figure 3. Typcial HPLC chromatogram of OPA derivatized amino acids obtained from a fresh sample of *A. tripedale* (sample No. TAX 6082, kindly provided by Dr. R. M. Fritsch, Gatersleben, Germany). Identified peaks are marked by the corresponding amino acids. Numbering of cysteine sulfoxides corresponds to **Figures 2** and **4**, and amounts related to the fresh weight of bulbs were also given (n = 3). *: peak also contains traces of serine.

a Shimadzu LC 20 HPLC system consisting of an autosampler, a high-pressure mixing pump, a column oven, an UV detector (334 nm) and a QTrap 2000 equipped with a TurboIonspray ion source (Applied Biosystems/MDS Sciex, Toronto, Canada). ESI-MS operating conditions for the qualitative analysis of the extracts were as follows: positive ionization mode, scan range 30−1000 amu; source temperature 200 °C; ion spray voltage 5,500 V; curtain gas setting 10; declustering potential 110 V; entrance potential 11 V; flow rate 0.25 mL/min (HPLC separation). The HPLC conditions were as follows: a 50 mmol ammonium acetate buffer pH 6.5/acetonitrile gradient with a constant flow rate of 0.25 mL was applied (A, 50 mmol ammonium acetate buffer pH 6.5; B, acetonitrile). Gradient: isocratic 78% A for 20 min; 78−75% A over 29 min; 75% A for 1 min; 75−71% A over 4 min; 71% A for 1 min; 71−68% A over 8 min; 68−63% A over 2 min; 63% A for 10 min; 78% A for 10 min. A 250/2 Nucleodur 100-5 C18 EC column (Macherey Nagel, Düren, Germany) was used for all experiments.

Extraction of Precursors 8, 9 and 10. Bulbs (30.3 g fresh weight) were sliced and given into 80 mL of methanol. After extraction under reflux for 10 min, the extract was cooled and homogenized. Another 40 mL of methanol was added, and extraction under reflux was continued for an additional 10 min. After filtration, the solvent was removed under reduced pressure at 30 °C. The obtained residue was resolved in 60 mL of OPA reagent, and 500 μL of *tert*-butylthiol was added. After 30 min of incubation in the dark, 1 mL of a methanolic iodoacetamide solution (1 mmol) was added. After 3 min, the solution was filtered through a 0.45 μm cellulose acetate filter. The solvent was evaporated under reduced pressure, and the residue was stored at −20 °C for further processing.

Extraction Procedure for Volatile Compounds 11, 12, 14 and 15. Bulbs (40.7 g fresh weight) were homogenized and placed in 250 mL of water. This extract was shaken for 30 min. Afterward, the water phase was extracted with 4 portions of 30 mL of ethyl acetate. The combined ethyl acetate layers were evaporated at 30 °C under reduced pressure until near dryness and rediluted in 3 mL of methanol. Samples were cooled down to 3 °C and analyzed immediately by HPLC−MS/MS.

Preparative Separation of Precursors 8, 9 and 10. A Waters HPLC system (600 E System controller and a Waters 991 PDA) was used for separation. Preparative HPLC was performed on a VP 250/21 Nucleodur 100-5 C18 EC column (Macherey Nagel). A methanol/ammonium acetate buffer 50 μM pH 6.5 gradient with a constant flow rate of 10 mL/min was used (A, methanol; B, ammonium acetate buffer). Gradient: isocratic 60% A for 10 min ; 60−55% A over 20 min; isocratic 55% A for 10 min; 55−30% A over 10 min; 30−10% A over 10 min; isocratic 10% A for 20 min. UV detection was performed at 334 nm. Substance **8** eluted at 58 min, substance **9** at 42 min and substance **10** at 50 min. Purity of fractions was checked by direct injections into the MS. The collected fractions of the cysteine sulfoxides were instantly dried under vacuum and stored at −20 °C. Obtained yields were **8**, 15 mg; **9**, 1,160 mg; **10**, 19 mg.

Structure Elucidation. ESI-MS/MS measurements, as necessary in combination with HPLC separation, were conducted using a Shimadzu LC 20 HPLC system containing autosampler, high-pressure mixing pump, column oven and UV-detector in combination with a QTrap 2000 equipped with a TurboIonspray ion source (Applied Biosystems/MDS Sciex, Toronto, Canada). The ESI-MS operating conditions for qualitative determination of OPA derivatized cysteine sulfoxides were positive ion mode or negative ion mode, scan range 30−1000 amu, source temperature 200 °C, ion spray voltage 5.5 kV, curtain gas 10, declustering potential 80 V or 110 V, entrance potential 10 V, flow rate 10 μL/min (direct infusion) or 0.25 mL/min (HPLC separation). ESI-MS operating conditions for fragmentation experiments were positive ion or negative ion mode, scan range 30−1000 amu, source temperature off, ion spray voltage 5.5 kV, curtain gas 10, declustering potential 110 V, entrance potential 10 V, collision energy setting 22−52; collision cell entrance potential 25.42 V, collision cell exit potential 3 V, flow rate 10 μL/min (direct injection). HR-ESI-MS experiments have been performed with a Micromass Autospec (Manchester, England).

NMR experiments have been conducted on a JEOL-ECA 500 spectrometer (Tokyo, Japan). By the aid of standard correlation experiments (COSY, TOCSY, HSQC, HMBC), structure elucidation of isolated compounds was performed.

The measurement of optical rotation was performed on a Jasco DIP-370 digital photometer (Zimmern, Germany) at 589 nm and 20 °C. The samples were dissolved in methanol. IR spectra were recorded on a Bruker Alpha-P FT-IR (Ettlingen, Germany). UV spectra were performed on a Shimadzu UV-2401 PC (Kyoto, Japan).

Analytical Data of the Identified Compounds. *OPA-Derivative of* (+)-*S*-(*1-Butenyl*)-L-*cysteine Sulfoxide* (***8***, *Figure* **4**). NMR data are given in **Table 1**. FT-IR [ν_{max}, cm^{-1}, intensity given as strong (s), medium (m) or weak (w)]: 453 (w), 547 (w), 613 (w), 636 (w), 733 (w), 748 (m), 809 (w), 847 (m), 963 (w), 1015 (s, S=O), 1118 (m), 1148 (w), 1164 (m), 1236 (m), 1287 (m), 1325 (m), 1365 (m), 1408 (s), 1456 (s), 1505 (w), 1549 (s, C=0), 1615 (w, C−N amide), 2874 (m, CH, CH_2), 2897 (m, CH, CH_2), 2929 (m, CH, CH_2), 2962 (m, CH, CH_2), 3050 (w), 3192 (m). UV (λ_{max} nm, methanol): 202, 231, 298, 337. ESI-MS/MS, positive ionization mode, m/z (relative intensity): 55 (20), 79 (8), 103 (16), 121 (16), 143 (8), 156 (16), 170 (100), 174 (28), 182 (36), 203 (84), 220 (16), 228 (20). HR-ESI-MS $[M + H]^+$: 380.1373; molecular formula $C_{19}H_{26}N_1O_3S_2$; MS calculated $[M + H]^+$ 380.1354. Specific optical rotation $[\alpha_D^{20}]$: −89.0 mL g^{-1} dm^{-1} (589 nm, in methanol).

OPA Derivative of γ-*Glutamyl*-(+)-*S*-(*1-butenyl*)-L-*cysteine Sulfoxide* (***9***, ***Figure* 4**). NMR data are given in **Table 1**. FT-IR [ν_{max}, cm^{-1}, intensity given as strong (s), medium (m) or weak (w)]: 451 (s), 488 (w), 619 (s),

655 (s), 884 (m), 934 (m), 1013 (s, S=O), 1266 (s, O−C), 1399 (s, O−C), 1541 (s, C=O), 1698 (s, C−N amide), 2210 (w), 2838 (m, CH, CH_2), 2915 (w, CH, CH_2), 3012 (m), 3183 (m). UV (λ_{max} nm, methanol): 203, 232, 284, 298, 336. ESI-MS/MS, positive ionization mode, *m*/*z* (relative intensity): 57 (8), 88 (8), 150 (15), 154 (4), 188 (4), 202 (15), 234 (31), 244 (25), 262 (100), 315 (8), 331 (27), 349 (37, 453 (19). HR-ESI-MS $[M + H]^+$: 509.1776; molecular formula $C_{24}H_{33}N_2O_6S_2$; MS calculated $[M + H]^+$ 509.1780. Specific optical rotation $[\alpha_D^{20}]$: −8.45 mL g^{-1} dm^{-1} (589 nm, in methanol).

OPA Derivative of γ-Glutamyl-S-(1-butenyl)-L-cysteine (***10***, ***Figure* 4**). NMR data are given in **Table 1**. FT-IR [ν_{max}, cm^{-1}, intensity given as strong (s), medium (m) or weak (w)]: 458 (w), 482 (m), 620 (m), 655 (m), 723 (w), 748 (w), 884 (w), 925 (w),1015 (m), 1047 (m), 1164 (w,), 1271 (s, O−C), 1403 (s, O−C), 1542 (s, C=O), 1647 (m, C−N amide), 2919 (w, CH, CH_2). UV (λ_{max}, methanol): 202, 230, 284, 297, 335. ESI-MS/MS, positive ionization mode, *m*/*z* (relative intensity): 150 (13), 159 (8), 176 (8), 216 (17), 234 (21), 244 (48), 262 (100), 419 (21), 437 (47), 439 (17). HR-ESI-MS $[M + H]^+$: 493.1819; molecular formula $C_{24}H_{32}N_2O_5S_2$; MS calculated $[M + H]^+$ 493.1831. Specific optical rotation $[\alpha_D^{20}]$: −71.0 mL g^{-1} dm^{-1} (589 nm, in methanol).

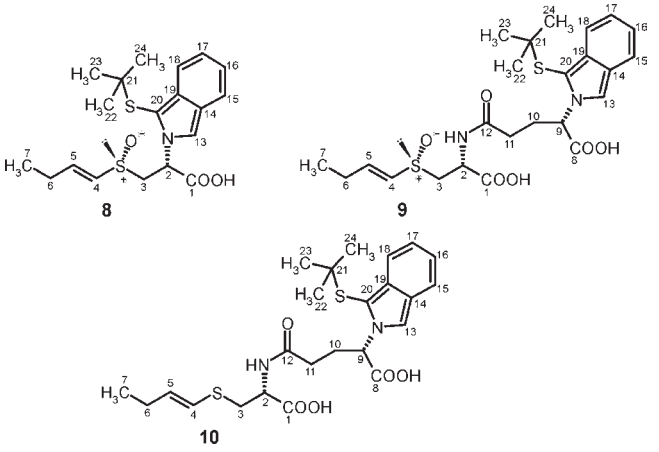

Figure 4. Newly described cysteine sulfoxides found in *A. tripedale*. Compounds have been isolated as the corresponding *o*-phthaldialdehyde derivatives (OPA). **8**: (+)-*S*-(1-Butenyl)-L-cysteine sulfoxide. **9**: L-γ-Glutamyl-(+)-*S*-(1-butenyl)-L-cysteine sulfoxide. **10**: L-γ-Glutamyl-*S*-(1-butenyl)-L-cysteine.

Di-(1-Butenyl)-disulfide (***11***, ***Figure* 5**). ESI-MS/MS, positive ionization mode, *m*/*z* (relative intensity): 45 (100), 55 (29), 71 (19), 85 (51), 87 (20), 88 (7) 119 (10), 175 (3). HR-ESI-MS $[M + H]^+$: 175.0626; molecular formula $C_8H_{15}S_2$; MS calculated $[M + H]^+$ 175.0615.

Di-(1-S-sulfoxymethyl-butyl)-disulfide (***12***, ***Figure* 5**). ESI-MS/MS, positive ionization mode, *m*/*z* (relative intensity): 87 (18), 119 (13), 151 (39), 175 (100), 239 (37), 285 (9), 303 (96). HR-ESI-MS $[M + H]^+$: 303.0628; molecular formula $C_{10}H_{23}O_2S_4$; MS calculated $[M + H]^+$ 303.0581.

1-S-Sulfoxymethyl-butyl-1′-S-sulfoxybutyl-butyl-disulfide (***14***, ***Figure* 5**). ESI-MS/MS, positive ionization mode, *m*/*z* (relative intensity): 39 (10), 41 (8), 45 (8), 47 (13), 55 (45), 77 (7), 85 (54), 87 (100), 89 (13), 119 (55), 151 (5), 175 (13), 345 (1). HR-ESI-MS sodium adduct $[M + Na]^+$: 367.0854; molecular formula $C_{13}H_{28}Na_1O_2S_4$; MS calculated $(M + Na]^+$ 367.0870.

1-S-Sulfoxymethyl-butyl-1′-S-sulfoxy-1-butenyl-butyl-disulfide (***15***, ***Figure* 5**). ESI-MS/MS, positive ionization mode, *m*/*z* (relative intensity): 39 (85), 41 (30), 55 (38), 69 (11), 85 (35), 87 (100), 119 (52), 151 (6), 175 (8), 343 (3). HR-ESI-MS sodium adduct $[M + Na]^+$: 365.0708; molecular formula $C_{13}H_{26}Na_1O_2S_4$; MS calculated $[M + Na]^+$ 365.0713.

RESULTS AND DISCUSSION

Origin of Plant Material and Usage. During botanical expeditions in the northwest parts of Iran between 2004 and 2007, *A. tripedale* was found as a fresh vegetable at nearly every local market in spring time. The material has a very strong smell and taste and is highly prized by the local population. There were no reports about direct use as a vegetable without cooking. However, the leaves were frequently used for flavoring bread. Dealers on the market have been asked for the origin of the material. It was told that *A. tripedale* was exclusively collected in the wild and sometimes transported over several hundred kilometers. Further it was told that the plant grows "nearby the rocks at a height over 2.000 m", but no exact places could be mentioned. The reason for this fact is that plant material was harvested by local plant collectors and was sold to dealers afterward, which organize

Table 1. ^1H NMR and ^{13}C NMR Data of Compounds **8**−**10** in Methanol-d_3[a]

	compd **8**		compd **9**		compd **10**	
C no.	^{13}C	^1H	^{13}C	^1H	^{13}C	^1H
1	173.2		177.7		176.9	
2	55.8	5.99 (dd, 1, *J* = 6.01, 15.18)	49.9	4.39 (dd, 1, *J* = 4.56, 9.16)	57.3	4.49 (dd, 1, *J* = 3.15, 7.16)
3	58.3	3.53 (dd, 1, *J* = 10.02, 13.46)	58.1	3.14 (dd, 1, *J* = 2.58, 9.16)	46.1	2.45 (dd, 1, *J* = 7.16, 13.46)
		3.85 (dd, 1, *J* = 4.87, 13.46)		3.31 (dd, 1, *J* = 2.58, 4.56)		3.65 (dd, 1, *J* = 3.15, 13,46)
4	129.3	6.08 (d, 1, *J* = 15.18)	131.1	6.46 (d, 1, *J* = 15.32)	122.5	5.96 (d, 1, *J* = 14.03)
5	144.6	6.32 (tt, 1, *J* = 6.01, 15.18)	145.2	6.57 (tt, 1, *J* = 6.30, 15.32)	132.7	5.65 (tt, 1, *J* = 6.87, 14.03)
6	24.9	2.00 (m, 2)	25.2	2.28 (m, 2)	25.9	2.02 (m, 2)
7	10.9	0.87 (t, 3, *J* = 7.45)	11.5	1.09 (t, 3, *J* = 7.16)	12.9	0.92 (t, 3, *J* = 7.45)
8			176.8		175.9	
9			70.0	5.71 (dd, 1, *J* = 5.15, 10.31)	60.4	5.67 (dd, 1, *J* = 5.73, 11.17)
10			31.2	2.30 (m, 1); 2.65 (m, 1)	30.5	2.30 (m, 1); 2.60 (m, 1)
11			33.1	2.15 (m, 1); 2.37 (m, 1)	32.7	2.22 (m, 1); 2,28 (m, 1)
12			177.3		176.3	
13	114.6	7.70 (s, 1)	114.8	7.70 (s, 1)	114.9	7.69 (s, 1)
14	125.0		125.1		125.4	
15	120.1	7.50 (d, 1, *J* = 8.31)	120.3	7.51 (d, 1, *J* = 8.59)	120.1	7.48 (d, 1, *J* = 8.31)
16	120.4	6.87 (dd, 1, *J* = 6.59, 8.31)	120.4	6.85 (dd, 1, *J* = 6.30, 8.59)	120.4	6.85 (dd, 1, *J* = 6.30, 8.31)
17	121.9	6.93 (dd, 1, *J* = 6.59, 8.31)	121.8	6.91 (dd, 1, *J* = 6.30, 8.59)	121.8	6.91 (dd, 1, *J* = 6.30, 8.59)
18	120.2	7.57 (d, 1, *J* = 8.31)	120.1	7.58 (d, 1, *J* = 8.59)	120.0	7.57 (d, 1, *J* = 8.59)
19	131.2		131.0		130.6	
20	110.2		109.2		111.5	
21	50.3		49.8		48.5	
22−24	30.3	1.23 (s, 9)	30.3	1.23 (s, 9)	32.0	1.42 (s, 9)

[a] Chemical shifts δ are given in ppm and coupling constants *J* in Hz.

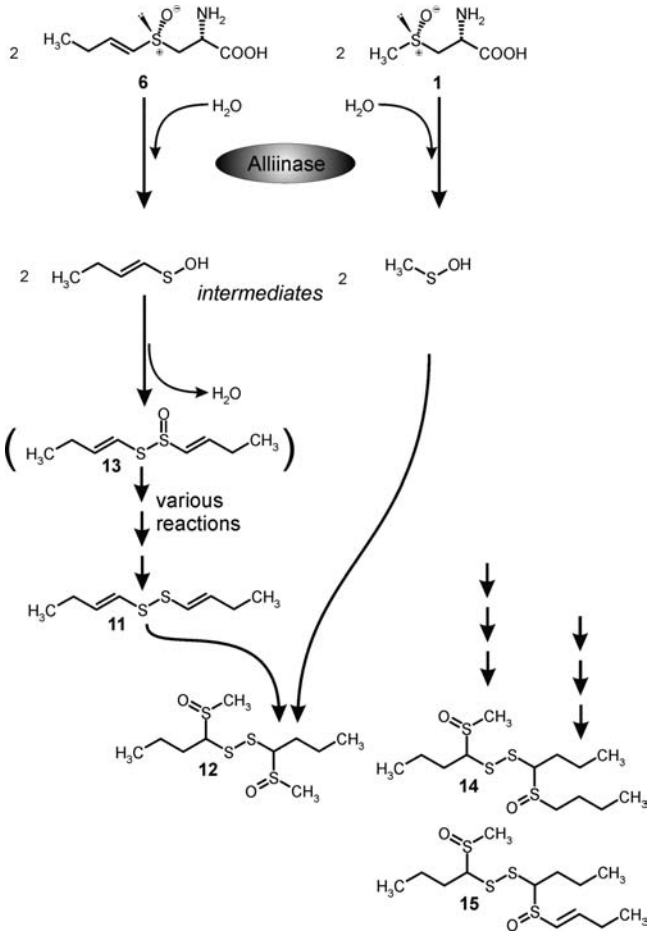

Figure 5. Hypothetical scheme of simultaneous alliinase cleavage of homoisoalliin (**6**) and methiin (**1**). Identified compounds were: **11**, di-(1-butenyl)-disulfide; and **12**, di-(1-*S*-sulfoxymethyl-butyl)-disulfide. Di-(1-butenyl)-thiosulfinate **13** could not be detected. Further on, 1-*S*-sulfoxymethyl-butyl-1′-*S*-sulfoxybutyl-butyl-disulfide **14** and 1-*S*-sulfoxymethyl-butyl-1′-*S*-sulfoxy-1-butenyl-butyl-disulfide **15** were found.

shipping of material to local markets. There, the material is probably sold to further local market dealers (**Figure 1c**). Original places of *A. tripedale* could be first identified after interviewing local plant collectors in mountain areas (**Figure 1a**). Plant material has been truly identified by Dr. Reinhard M. Fritsch, IPK Gatersleben, Germany.

Because the characteristic flavor occurs after disrupting plant tissues, it was assumed that the flavor formation is based on the alliinase reaction. Out of this hypothesis, plant must contain cysteine sulfoxides, which are subjected to alliinase after disruption of cells. In a first step of investigation, an enzyme inactivated extract was derivatized by a modified OPA reagent (*10*) and amino acid derivatives have been analyzed by HPLC−MS/MS (**Figure 3**). Besides known amino acids and cysteine sulfoxides like methiin (**1**, t_R = 18.86 min) and butiin (**5**, t_R = 62.00 min), also some undescribed compounds could be identified (peaks at t_R 8.93 min, 45.00 and 59.79 min). Peaks of known amino acid derivatives have been identified by mass spectra as well as authentic reference compounds. Because of the somewhat eye irritating effect of a plant extract, it was assumed that new amino acid derivatives could be related to isoalliin (**4**), which is not present in *A. tripedale*. From the known cysteine sulfoxides, **1** was found in a concentration of 0.44% related to the fresh weight. This compound has been also obtained in nearly all investigated *Allium* species, mostly in higher concentrations (*10*, *12*).

Isolation and Identification of the Precursor (+)-*S*-(1-Butenyl)-L-cysteine Sulfoxide 8. In order to elucidate molecules involved in the formation of the aroma components of *A. tripedale*, also OPA derivatized amino acids have been separated in preparative scale. First, isolation of cysteine sulfoxides without precolumn derivatization was tried. However, separation of pure compounds failed because of the complexity of the extract.

Also the preparative purification of the OPA derivatives turned out to be difficult. In a first set of experiments, isolated compounds decomposed rapidly. Two reasons were found for this: (i) addition of iodoacetamide after OPA addition is absolutely necessary in order to trap a surplus of OPA reagent; (ii) evaporation of solvent must be performed at the lowest possible temperature not exceeding 30 °C.

The derivatization procedure was first examined and optimized with synthetic L-(+)-alliin. Structure elucidation of **8**−**10** is mainly based on NMR analysis using ^{1}H, ^{13}C, COSY, HMBC and HMQC experiments. Results of structure assignment are summarized in **Table 1**. The numbering of carbon atoms correlates to **Figure 4**. Experiments with the synthetic alliin have been used for safe determination of chemical shifts at positions 1−3 (cysteine moiety) and 13−24 (OPA moiety), which should be nearly identical for all cysteine sulfoxides. All shifts for the aromatic protons of the OPA moiety are in the range of δ 6.85 to δ 7.58 and therefore clearly separated from all other signals of the molecule. The *tert*-butyl residue (21−24) gives a sharp signal at δ 1.23, which also does not interfere with further signals of the obtained proton spectrum.

The 1-butenyl residue is characterized by an aliphatic double bond (C4, δ 129.3; H4, δ 6.08; C5, δ 144.6; H5, δ 6.32), a methylene group (C6, δ 24.9; H6, δ 2.00) and a terminal methyl group (C7, δ 10.9; H7, δ 0.87). The protons H5 and H6 showed a coupling constant of J = 15.18 Hz clearly indicating *trans* configuration, which is identical with isoalliin (*13*, *14*).

One further question is the configuration of the sulfoxy group. Optical rotation does not supply a sufficient answer, because derivatization of the amino function at C2 resulted in a strong shift toward negative values. However, as it could be demonstrated for synthetic L-(+)-alliin and L-(−)-alliin, proton shifts at C3 are strongly influenced by the sulfoxy group in the neighboring position (*11*). In the case of (+)-configuration, both protons at C3 are clearly separated from each other forming a doublet−doublet spin system. In the case of (−)-configuration, both protons have nearly the same chemical shift of about δ 3.4. Substance **8** showed definitely separated proton signals at δ 3.53 and δ 3.85 proving (+)-configuration of the sulfoxy group. The results are also in accordance with published data of Van den Broek (*15*) and Kubec (*16*).

Further on, the IR spectrum of **8** showed the expected strong sulfoxide band at 1015 cm^{-1}. This also supports (+)-configuration. In the case of (−)-sulfoxide, this band has a maximum at about 1000 cm^{-1} (*11*). Carboxy and amide functionalities are given by bands at 1456 cm^{-1}, 1549 cm^{-1} and 1615 cm^{-1}.

The molecular formula of **8** was confirmed by HR-MS and MS/MS experiments. In an alternative experiment, a methanolic extract before OPA derivatization was directly analyzed by MS/MS. Compound **6**, which corresponds to **8**, gave a characteristic signal at m/z 88 $[M + H]^+$ (base peak) in the positive ionization mode. In the negative mode, the fragment at m/z 103 is very characteristic indicating the 1-butenyl-sulfoxide residue formed by a heterolytical cleavage of the bond between the sulfur atom and C3. Compound **6** was named homoisoalliin, because it is homologous to isoalliin plus one methylene group.

Identification of the Precursor L-γ-Glutamyl-(+)-*S*-(1-butenyl)-L-cysteine Sulfoxide 9. As a second precursor, the relatively stable

L-γ-glutamyl-(+)-*S*-(1-butenyl)-L-cysteine sulfoxide (**9**) could be detected and separated as an OPA derivative. **9** was found to be the main cysteine sulfoxide of the *A. tripedale* sample TAX 6082 as shown in **Figure 3**. This is rather interesting, because γ-glutamyl cysteine sulfoxides cannot be subjected directly to alliinase. It can be assumed that **9** is the storage form of homoisoalliin (**6**).

Again, structure elucidation was mainly based on two-dimensional NMR techniques. The shifts of the aromatic protons of the OPA moiety were comparable to the shifts at compound **8** and have been observed in a range between δ 6.8 and δ 7.7. The shift of the *tert*-butyl protons also matched the NMR data of compound **8**. The protons of the cysteine moiety (H2, δ 4.39; H3, δ 3.14; H3′, δ 3.31) were shifted to higher field in comparison to **8**. This indicates a variation at this part of the molecule.

The γ-glutamyl moiety was characterized by the two methylene groups (C10, δ 31.2; H10, δ 2.30, δ 2.65; C11, δ 33.1; H11, δ 2.15, δ 2.37) as well as the chiral position 9 (C9, δ 70.0; H9, δ 5.71). Two additional carboxy groups have been also observed (C8, δ 176.8; C12, δ 177.3). The above-described data set was found in addition to that of **8** strongly supporting a γ-glutamyl residue. This structural element is also very characteristic for the genus *Allium* (*9*).

All other chemical shifts were rather similar. The 1-butenyl residue was characterized by an aliphatic double bond (C4, δ 131.1; H4, δ 6.46; C5, δ 145.2; H5, δ 6.57), a methylene group (C6, δ 25.2; H6, δ 2.28) and the terminal methyl group (C7, δ 11.5; H7, δ 1.09). The coupling constant of 15.32 Hz between H5 and H6 indicated *trans* configuration (*13*, *14*). Again, the configuration of the sulfoxy group was determined by comparison with data from synthetic reference material as described above (*9*). The shifts of the protons at C3 (δ 3.14 and δ 3.31) gave well separated doublet−doublets clearly indicating a (+)-configuration of the sulfoxy group. In addition, the IR spectrum of **9** showed the expected strong (+)-sulfoxide band at 1015 cm^{-1}. The IR bands at 1399 cm^{-1}, 1541 cm^{-1} and 1698 cm^{-1} resulted from the carboxy and amide functionalities.

As described for **8**, also MS/MS experiments could be performed with **9** prior to OPA derivatization. The already mentioned fragments of **6** have been also found. The two expected structural moieties, the cysteine residue and the 1-butenyl-sulfoxide residue, have been characterized by two intensive signals at *m*/*z* 88 $[M + H]^+$ (88% relative intensity) and *m*/*z* 103 $[M]^-$ (68% relative intensity). The obtained substance can be named as γ-glutamyl-homoisoalliin.

Identification of the Precursor L-γ-Glutamyl-(+)-*S*-(1-butenyl)-L-cysteine 10. As a third precursor, the relatively unstable L-γ-glutamyl-(+)-*S*-(1-butenyl)-L-cysteine was detected and separated as an OPA derivative (**10**). Structure elucidation was mainly performed by NMR techniques. ^1H and HMQC measurements clearly showed the OPA moiety as given in **Table 1**. The slight low-field shift of the *tert*-butyl protons (δ 1.42) might result from an altered conformation of the whole molecule due to the nonoxidized sulfur atom. The protons of C3 (H3, δ 2.45; H3′, δ 3.65) showed a significant high-field shift in comparison to **8** and **9**, whereas the proton at C2 is less affected (H2, δ 4.49). Also signals belonging to positions 4 and 5 are shifted to higher field (C4, δ 122.5; H4, δ 5.96; C5, δ 132.7; H5, δ 5.65). Again, the coupling constant between H5 and H6 was relatively large (J = 14.03 Hz) indicating *trans* configuration. The ^{13}C and ^1H signals assigned to the γ-glutamyl residue were comparable to **9**.

In comparison to **9**, these altered chemical shifts close to sulfur atom indicate significant changes in this part of the molecule. A reduced mass of 16 amu and a relatively weak signal at 1015 cm^{-1} in the FT-IR spectrum indicates **10** as desoxy-cysteine sulfoxide (*11*). Reanalysis of **10** by FT-IR after some weeks of storage showed a strongly increased intensity of the sulfoxy band at 1015 cm^{-1}. This substance is obviously rather sensitive to oxidation. γ-Glutamyl-desoxy-cysteine sulfoxides are proposed to be the precursors of cysteine sulfoxides (*9*). Interestingly, oxidation to the (+)-isomer seems to be preferred as given by the IR spectrum because no band was visible at about 1000 cm^{-1} indicating the (−)-isomer or a racemate.

The desoxy-structure of **10** could be also identified by MS/MS measurements. In negative ionization mode of **10** prior to OPA derivatization, no fragment with a *m*/*z* of 103 $[M^-$, butenyl-sulfoxy] could be detected. Instead, a fragment with an *m*/*z* of 87 $[M^-$, butenyl sufide] was observed. This fragment can be explained by a heterolytical cleavage of a thioether between the sulfur atom and C3. The isolated compound can be named as γ-glutamyl-desoxyhomoisoalliin. The structure of desoxyhomoisoalliin is also given in **Figure 1** (**7**).

Table 2. Content of Sulfur Compounds (Precursors) of *A. tripedale* and *A. siculum*[a]

			rel amts if individual compounds [%]				
sample	total amount [%]	SD [%]	**1**	**5**	**8**	**9**	**10**
TAX 6082	1.44	±0.019	31	1	6	60	3
Acc 1157	0.30	±0.003	49	7	22	nd	22
Acc 1173	0.51	±0.056	40	5	17	nd	38
Acc 1186	0.71	±0.005	38	4	7	nd	51
TAX 0098	1.59	±0.029	22	3	23	50	3

[a] Presursors were determined as OPA derivatives. The numbering of compounds corresponds to **Figures 2** and **4**. Given values are related to the fresh weight of bulbs. TAX 6082: *A. tripedale*, IPK collection. Acc 1157, Acc 1173 and Acc 1186: *A. tripedale*, samples from Iran. TAX 0098: *A. siculum*, IPK collection. SD, standard deviation; nd, not detected.

Quantitative Determination of Precursors. Results of quantitative determinations of precursors, mainly cysteine sulfoxides, are summarized in **Table 2**. Investigated material was either collected in Iran (Acc numbers; compare **Figure 1**) or obtained from the Living Plant Collection of IPK, Gatersleben, Germany (TAX numbers). All compounds were analyzed as their corresponding OPA derivatives.

Methiin (**1**) as well as butiin (**5**) is present in all samples. The newly discovered homoisoalliin (**6**, **8**) was also present in all samples and was found in relative concentrations between 6% (TAX 6082) and 22% (Acc 1157), related to total amount of compounds. Interestingly, **9** was missing in all samples collected in Iran (Acc 1157, Acc 1173 and Acc 1186). The highest total amount of precursors was found in sample Tax 6082 (1.44%), which is comparable to the sample of *A. siculum* (TAX 0098, 1.59%).

Due to the relatively high amount of cysteine sulfoxides, the taste of *A. tripedale* should be much more pungent than the taste of *A. cepa* (*17*). The γ-glutamyl derivative **9** (precursor of **8**) showed the highest concentration in the sample TAX 6072, which was freshly obtained from IPK Gatersleben and analyzed directly. Samples from Iran were analyzed some weeks after collection probably resulting in a decreased amount of **9** (amounts below detection limit). During storage, homisoalliin (**6**, **8**) seems to be liberated out of the γ-glutamyl derivative resulting in an increased pungency. Consequently, *A. tripedale* samples Acc 1157 and Acc 1173 showed the highest relative concentrations of homoisoalliin.

The desoxy derivative **10** exhibited the highest concentrations in all three samples from Iran (Acc 1157, Acc 1173, Acc 1186). Compound **10** is a precursor of **9** and also of **8**. Samples in Iran were collected in early spring time, whereas the samples from IPK

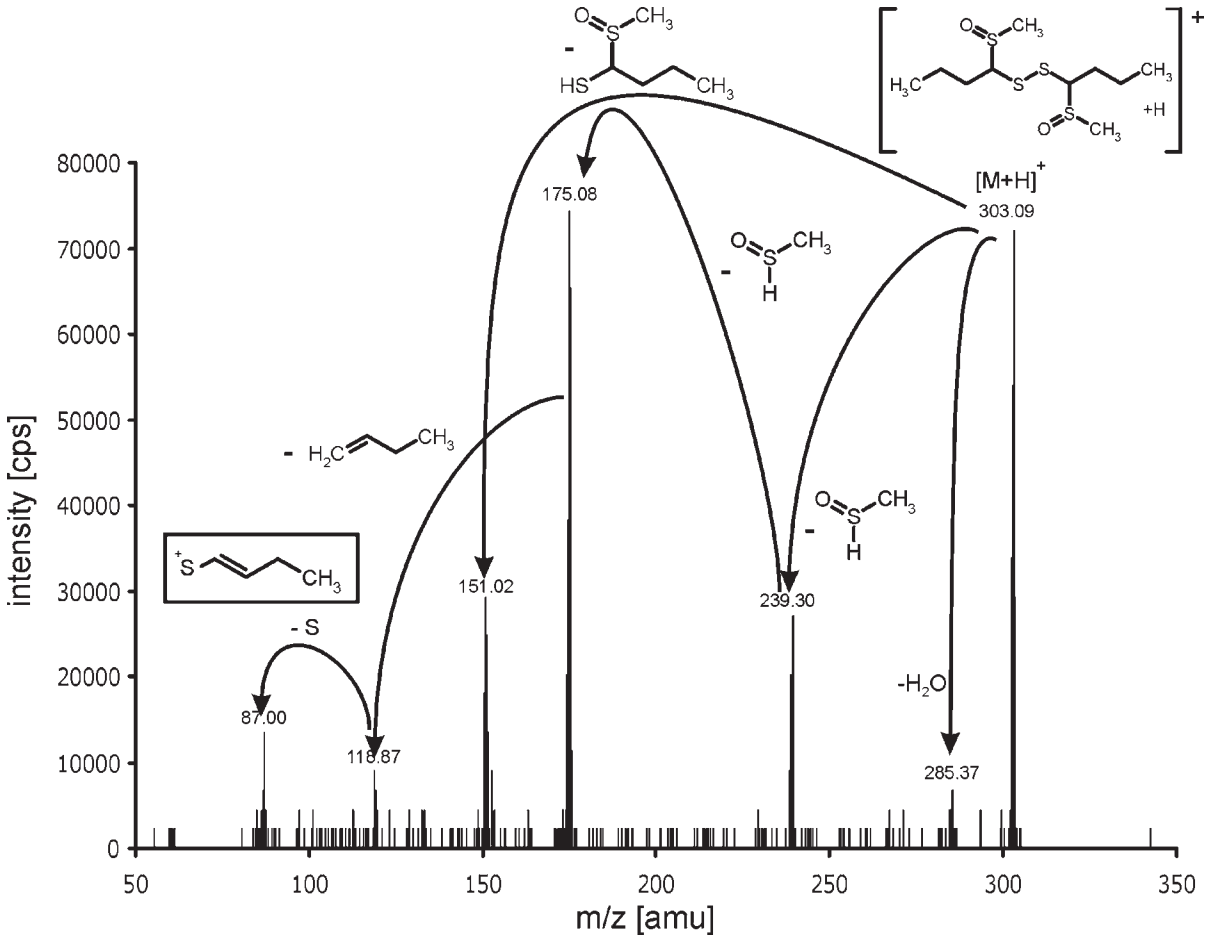

Figure 6. MS-fragmentation scheme of compound **12**. The structure of the resulting fragment at *m*/*z* 87 amu (1-butenylsulfide cation) is given inside the box.

Gatersleben were harvested after flowering of plants. It can be assumed that the relative composition of sulfur compounds strongly depends on time of harvest. As another interesting observation, *A. tripedale* (TAX 6082) and *A. siculum* (TAX 0098), which were grown at the same place and harvested at the same time, exhibited a very similar pattern of precursors.

Isolation and Identification of Di-1-butenyl Disulfide 11 and Cepaene-like Enzymatic Products (12, 14, 15). For determination of alliinase products, bulb material was crunched to allow digestion of cysteine sulfoxides. After 30 min, enzymatic products were extracted by ethyl acetate and were subjected to HPLC−MS/MS. The obtained yields were extremely low (below 1 mg) so that analysis was only possible by MS/MS experiments and HR-MS. Obtained mass spectra have been selectively screened for a fragment ion at 87 amu in positive ionization mode representing a butenylthio moiety. This strategy was chosen to find specifically alliinase products of homoisoalliin **6**. About 14 substances could be detected by a fragment ion at 87 amu. Out of this, the structure of the following four compounds could be tentatively elucidated. This MS-based structure elucidation is justified, because (i) precursors of the alliinase reaction are known, (ii) the mechanism of the alliinase reaction is well described and (iii) formation of volatile compounds is well investigated (*3*). Compounds **12**, **14**, and **15** are in full accordance with these above-mentioned points.

As shown in **Figure 5**, cysteine sulfoxides, mainly methiin **1** and homoisoalliin **6**, were subjected to the alliinase reaction. Because of the structural similarity of isoalliin and homoisoalliin **6**, an *A. cepa*-like chemistry can be expected for the enzymatic products (*3*). Interestingly, only the disulfide **11** and cepaene-like compounds **12**, **14**, **15** could be identified. Because all possible precursors of the alliinase reaction could be identified by HPLC as shown in **Figure 3**, structure elucidation was based on various MS experiments.

Confirmation of the proposed molecular formulas was made by HR-MS giving differences of only 0.2 to 4.7 mDa to the calculated masses. MS/MS experiments have been performed to achieve full structure elucidation. Compound **11**, a butenyl disulfide, was found to be the mother compound of **12**, **14** and **15**. For **11**, the main fragment was found at *m*/*z* 45 in the positive ionization mode, representing the HCS^+ ion, which is typical for dibutyl disulfides. Further on, loss of one 1-butenyl residue gave the fragment *m*/*z* 119 $[M]^+$. The mass signals *m*/*z* 85, 87, and 88 $[M]^+$ are formed by different hetero- and homolytical cleavages of the disulfide bridge of **11** and also result from the above-described fragment at *m*/*z* 119 $[M]^+$. The mass signal at *m*/*z* 59 $[M + H]^+$ indicates a $C_2H_3S^+$ fragment, and *m*/*z* 55 $[M]^+$ with a relative intensity of 29% is characteristic for the 1-butenyl residue.

Compound **12** is formed by the reaction of two methylsulfenic acids with **11** in a cepaene-like manner (*3*). **Figure 6** shows the fragmentation pattern of **12**, which is characterized by two fragmentations with a loss of 64 amu each, representing the two methyl-sulfoxide moieties. According to the formation mechanism of cepaenes (*3*), these moieties are attached to positions 1 and 1′ of the dibutyl-disulfide backbone of **12**. The resulting disulfide at *m*/*z* 175 $[M + H]^+$ is identical with compound **11**. Cleavage of the disulfide bridge of **12** gave *m*/*z* 151 $[M]^+$. The fragment *m*/*z* 87 $[M]^+$ represented the butenyl sulfide structure.

Formation and fragmentation patterns of compounds **14** and **15** can be explained analogously to compound **12**. One methyl-sulfoxy

group is replaced either by a butyl-sulfoxy (**14**) or a butenyl-sulfoxy (**15**) moiety. Again, cleavage of both sulfoxy moieties resulted in m/z 175 $[M + H]^+$. Intensity of fragments larger than 175 amu was found to be very low. Analogously to substances **11** and **12**, the above-described fragments at m/z 151 [1-*S*-methyl-sulfoxy-butyl sulfide]$^+$, m/z 119 [butenyl disulfide]$^+$, m/z 87 [butenylthio]$^+$, 85 $[C_4H_5S]^+$, m/z 55 $[C_4H_7]^+$ and 45 $[HCS]^+$ could be detected. Further fragments at m/z 39 $[M]^+$ and m/z 41 $[M]^+$ have been observed, which can be explained by the fragmentation of the butyl and 1-butenyl residues. The much higher intensity of the fragment m/z 39 $[M]^+$ for compound **15** (85% relative intensity instead of 10% for **14**) underlines the thesis that compound **15** contains two butenyl moieties in total. One key fragment of **14** is at m/z 89 $[M]^+$ representing a butylsulfide ion, which is not visible in the MS of **15**.

Proposed Biogenetic Pathway of Di-(1-*S*-sulfoxymethylbutyl)-disulfide. The hypothetical biogenetic pathway for the formation of cepaene-like compounds is shown in **Figure 5**. After disruption of the cell, an enzyme with a C−S lyase activity (alliinase reaction) converts the precursors methiin (**1**) and homoisoalliin (**6**) into the corresponding sulfenic acids. As demonstrated in previous investigations (*10*), substrate specificity of alliinase for defined cysteine sulfoxides shows high variations. Highest activity was frequently found toward isoalliin (**4**), whereas methiin is converted with poor rates. We assume that also homoisoalliin (**6**) is converted rapidly by alliinase, because the typical odor occurs immediately after plant disruption. However, methiin (**1**) is the main alliinase precursor and, after some time, a surplus of metabolites coming from methiin will accumulate in disrupted plant tissue.

Coming back to the metabolism of homoisoalliin (**6**): the intermediate sulfenic acid will spontaneously dimerize to give di-1-butenyl thiosulfinate (**13**). However, this thiosulfinate could not be identified in the ethyl acetate extract. Alternatively it can be proposed that a sulfoxide is formed after enzymatic cleavage, which is homologous to the "lachrymatory factor" (*3*). But also a selective screen for this compound was without success.

Analogously to the chemistry of volatile compounds of *A. cepa* (*3*), thiosulfinates as well as the "lachrymatory factor" were rather quickly converted to the corresponding disulfides like di-1-butenyl disulfide (**11**), which was found in significant amounts. But also this compound is highly reactive because of the two double bonds of the two butenyl groups.

Coming back to the metabolite of methiin (**1**): because of the much slower alliinase reaction toward this compound and the surplus of methiin (**1**), molecules of methylsulfenic acid as a highly reactive intermediate can be added to the double bond of the already formed di-1-butenyl disulfide (**11**) in a cepaene-like manner to give di-(1-*S*-sulfoxymethylbutyl)-disulfide (**12**). This compound would sufficiently explain the rather strong and unpleasant smell of *A. tripedale*. Compounds **14** and **15** were formed in a very similar way. For **14**, the cysteine sulfoxide butiin (**5**) is necessary as precursor, which was found in rather low amounts in the fresh bulb (0.01%). In separate investigations it was found that alliinase from *Allium sativum* metabolizes **5** poorly.

These results raise many questions: Does the alliinase of *A. tripedale* react in the same manner as the alliinase known from *A. cepa*? Does a "lachrymatory factor" occur under certain conditions? Are there further volatile compounds, which are homologous to volatile compound from *A. cepa* like further cepaenes, zwibelanes and dithiethane-*S*-oxides? And what bioactivities do isolated compounds show? These are subjects for further investigations.

Conclusion. *A. tripedale* seems to have a rather similar chemistry to common onion, *A. cepa*. Both species have been used as vegetable, spice or even medicine. Isoalliin is the most important cysteine sulfoxide of *A. cepa*, whereas *A. tripedale* contains the homologous compound homoisoalliin, which is reported in nature for the first time. But already the Finnish Nobel Laureate Artturi Ilmari Virtanen predicted this compound in nature over 40 years ago (*18*). The now presented work is the proof of the theory of Virtanen. Additionally, the corresponding γ-glutamyl derivatives were described for both species.

Further on, the homologues of the 'lachrymatory factor' (thiopropanal *S*-oxide), which is well-known from *A. cepa* (*19*), can be expected in *A. tripedale*. Probably due to its instability, we were not able to isolate this compound. Instead of this, compounds homologous to cepaenes, which are also well-known from *A. cepa* (*3*), have been found. Described similarities between the chemistry of *A. cepa* and *A. tripedale* are unique in nature and are helpful for the understanding of the complex sulfur chemistry of the genus *Allium*.

Despite the small differences in the chemistry of *A. cepa* and *A. tripedale*, both plants have a completely different smell and taste. The smell and taste of *A. tripetale* are absolutely unique and not comparable to those of other *Allium* species. Basically, taste is very pungent with a strong sulfur note. These findings might give new input to plant breeders. It is also thinkable that carefully dried bulbs and leaves of *A. tripedale* can be merchandized as a spice also in the Western World.

As a last aspect of these investigations, it could be demonstrated that the sulfur chemistry of *Allium* species located in South West and Middle Asia is much more complex and diverse than the chemistry of those species which were traditionally used as vegetable, spice or medicine in the Western World. *Allium* species from Asia seems to be an excellent source for new sulfur compounds and aroma constituents as it also was reported previously (*20*, *21*). These findings should have a significant impact on plant breeders, especially those located in Asia.

ABBREVIATIONS USED

OPA, *o*-phthaldialdehyde; COSY, correlated spectroscopy; TOCSY, total correlated spectroscopy; HMQC, heteronuclear multiple-quantum coherence; HMBC, heteronuclear multiple-bond coherence; MS, mass spectrometry; ESI, electrospray ionization; HR-MS, high resolution mass spectrometry; HPLC, high performance liquid chromatography; FT-IR, Fourier-transformation infrared; nd, not detected; SD, standard deviation; t_R, retention time.

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