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ARTICLE

Isolation and Identification of a New Cysteine Sulfoxide and Volatile Sulfur Compounds from *Allium* Subgenus *Melanocrommyum*

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A new cysteine sulfoxide containing a pyridyl residue was identified in bulbs of *Allium* L. species belonging to the subgenus *Melanocrommyum* section *Megaloprason*. One of these species, *Allium stipitatum*, is widely used as a crop plant and in folk medicine in Central Asia. The new cysteine sulfoxide was identified as L-(+)-*S*-(2-pyridyl)-cysteine sulfoxide. Aside from this cysteine sulfoxide, several pyridyl compounds could be identified, which were formed out of cysteine sulfoxides by the action of alliinase. Found cysteine sulfoxides and their metabolites are chemically unstable; thus, the analysis is rather difficult. By combining high-performance liquid chromatography (HPLC), HPLC tandem mass spectrometry, NMR, IR, and photometric methods, full structure elucidation of the cysteine sulfoxide was possible. Alliinase reaction products were mainly determined by various MS techniques. The achieved results give new insights in the chemistry of *Allium* crop plants and are probably useful for chemotaxonomical classification of the subgenus *Melanocrommyum*.

KEYWORDS: ***Allium stipitatum*; *Allium altissimum*; *Melanocrommyum*; HPLC; L-(+)-*S*-(2-pyridyl)-cysteine sulfoxide; volatile sulfur compounds**

INTRODUCTION

The genus *Allium* L. has a large diversity, and more than 700 species worldwide are described until now. Nearly all of them occur in the semiarid regions of Europe, North America, North Africa, and Asia. The most common *Allium* species are garlic (*Allium sativum* L.) and onion (*Allium cepa* L.). These two species have been used as foods and spices since ancient times. Aside from their importance for nutrition, they have known pharmaceutical values. Remarkable are the antidiabetic and antithrombotic effects of *A. sativum* L. (*1*, *2*). The described effects are correlated to several sulfur compounds (*3*, *4*). Further important substances found in *Allium* species are flavonoids and steroid saponins. Steroid saponins are common in species of the families *Liliaceae* and *Alliaceae* (*11*). The most common types of steroid saponins are furostanol and spirostanol derivatives, which can be found in an amount of 0.3−1.1% related to the dry weight in *A. sativum* L. (*12*). Flavonoids like quercetin were observed in the whole genus *Allium*. In red bulbs of *A. cepa*, the concentration of flavonoids can be up to 2.1% related to the dry weight (*13*).

Allium species of the subgenus *Melanocrommyum* exhibit a great variability (> 200 species are known until now), and the center of distribution is in Middle and in Southwest Asia. Countries with a high diversity of this subgenus are Iran, Tajikistan, and Uzbekistan. Some of these species are known as ornamental plants in the western hemisphere, the so-called "drumstick onions". Genetic analysis displayed a high similarity of most of these ornamental plants. These species show a high polymorphism. By using genetic analysis, these plants could be related to the subgenus *Melanocrommyum* (*16*).

In Middle Asia, *Allium* species of the subgenus *Melanocrommyum* have a large range of usage. The characteristic smell and taste make the bulbs and leaves of these plants favored vegetables of the native populations. In several cases, the amounts of cysteine sulfoxides and their metabolites are rather high, so that the plants are used as spicy vegetables or are even not edible. *Allium stipitatum* Regel is a very common edible *Allium* species in Central Asia and is intensively used by local populations as a spicy vegetable and medicinal plant. In Iran, the plant is named "Musir", and in countries of the former Soviet Union, it is known as "Anzur". Beside *A. stipitatum*, several species of the subgenus *Melanocrommyum* are used in folk medicine. As an example, leaves and bulbs of *Allium severtzovioides* R.M. Fritsch are applied against stomach and duodenum diseases (*5*). *Allium motor* Kamelin et Levichev leaves are served as a tonic soup. *Allium komarowii* Lipsky is used as an anabolic for horses. Beside these extraordinary uses, it is applied against anemia and bad blood circulation. *Allium suworowii* Regel is used against early forms of bronchitis and tuberculosis. Studies peformed in our working group showed a very high antioxidative effect of these species (*7*).

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A number of species of the subgenus *Melanocrommyum* develop a very characteristic coloration. By cutting an onion and damaging the cells, an orange to red dye appears after some minutes. This red dye is a metabolite of L-(+)-*S*-(3-pyrrolyl)-cysteine sulfoxide, a newly discovered cysteine sulfoxide (*6*). This substance can be found in nearly all species of the subgenus *Melanocrommyum* but mostly in low concentrations. However, some species of the subgenus *Melanocrommyum*, section *Megaloprason*, do not contain this pyrrolyl-cysteine sulfoxide. Especially bulbs of *A. stipitatum* Regel and *A. altissimum* Regel showed no coloration after cutting (*8*). In the bulbs of *A. stipitatum* Regel and *A. suworowii* Regel, new steroid saponins of the spirostan series were isolated and identified (*14, 15*), but no reports about unusual cysteine sulfoxides were found. However, the alliinase activity of *A. stipitatum* Regel was found to be similar to other *Allium* species. The pH optimum (pH 7.5) and the temperature optimum (38 °C) were typical values (*17*).

Beside these findings, O'Donnell et al. suggested three new volatile compounds in samples of *A. stipitatum*, which showed antimicrobial activity (*9*). These compounds were identified as *N*-oxides, also described for basidomycetes (*10*). Extracts showed an activity against *Mycobacterium tuberculosis*. An explanation for the formation of these three new compounds is an alliinase-catalyzed reaction of an unknown cysteine sulfoxide. This hypothetical sulfoxide should contain a pyridine ring. According to O'Donnell et al., this pyridine ring should be substituted with a cysteine sulfoxide at position 2. The formation of these volatile compounds by an alliinase reaction insists that the main metabolites are sulfoxides, not pyridine-*N*-oxides. The aim of the now described work is the identification and structure elucidation of this hypothetic cysteine sulfoxide as well as investigation of direct metabolites of the alliinase reaction.

MATERIALS AND METHODS

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

Derivatization Reagent [*ortho*-Phthaldialdehyde (OPA) Reagent]. Under magnetic stirring, 140 mg of OPA was solubilized in 5 mL of methanol. Afterward, 200 μL of *t*-butylthiol was added. Then, 50 mL of borate buffer, pH 9.5, was slowly mixed with the OPA solution. The reagent was stored for at least 12 h under light protection before usage.

Plant Material. Plant material (*A. stipitatum* Regel) for structure elucidation was obtained from the living plant collection at IPK Gatersleben, Germany. The plant material for the quantitative analysis was collected in various regions of Middle Asia. *A. stipitatum* Regel samples 1086/2, 1090/2, and 1240 Z1 were collected in Iran. *A. stipitatum* samples 4238/1 and 4238/2 were collected in Turkmenistan. The bulbs of *Allium altissimum* Regel listed as samples 4206/1 and 4206/2 were collected in Uzbekistan. Voucher species were planted in the living *Allium* collection at IPK Gatersleben. Taxonomic determination of used plant material was done by Dr. R. M. Fritsch of the same institute.

Plant Processing and Instrumental Setup for Qualitative and Quantitative Analysis of Cysteine Sulfoxides. Bulbs were cleaned and sliced into pieces of 200−800 mg weight. Pieces were instantly placed in 20 mL of methanol to inhibit enzymatic activity and heated under reflux. After 10 min, the pieces were homogenized, and extraction was continued with a mixture of methanol/water (VT 1:1) by heating under reflux. Afterward, the extract was filtered, and the solvent was carefully evaporated under reduced pressure. The obtained completely dried extracts were stored in the freezer at −20 °C. For measurements, extracts were solubilized in 4.93 mL of OPA reagent and 50 μL of 2-methylpropanethiol. After a derivatization for 30 min in darkness, 20 μL of iodacetamide was added.

For quantitative measurements, 200 μL of samples was diluted in 800 μL of water. For qualitative measurements, 500 μL of samples was diluted in 500 μL of water. The diluted samples were injected either into the high-performance liquid chromatography (HPLC) or the HPLC MS/MS device. The HPLC system used for quantitative analysis was a Merck Hitachi 7000 series HPLC system. A VP 250/4 Nucleodur 100-5 C18 EC column (Macherey Nagel, Düren, Germany) was used for all HPLC measurements. The column oven was set to 30 °C. Calibration of the system was done with an OPA derivatized L-(+)-alliin standard. Chromatographic conditions were as follows: A phosphate buffer pH 6.5/ acetonitrile gradient with a constant flow rate of 1 mL was used (A, phosphate buffer, pH 6.5; B, acetonitrile): 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; and 78% A for 10 min; UV detection at 334 nm.

For qualitative HPLC-electrospray ionization (ESI)-MS, measurements were performed on a Shimadzu LC 20 HPLC system containing an autosampler, a high-pressure mixing pump, a column oven, and an UV detector in combination with 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, 30−500 amu; source temperature, 200 °C; ion spray voltage, 5.5 kV; curtain gas, 10; declustering potential, 110 V; entrance potential, 11 V; and flow rate, 0.25 mL/min (HPLC separation). The LC conditions were as follows: a 50 μM ammonium acetate buffer, pH 6.5/acetonitrile gradient with a constant flow rate of 0.25 mL was used (A, ammonium acetate buffer, pH 6.5; B, acetonitrile): gradient for quantitative analysis was also used for qualitative HPLC/MS experiments; UV detection at 334 nm. A 250/2 Nucleodur 100-5 C18 EC column (Macherey Nagel) was used for all experiments.

Plant Processing for MS Fragmentation Experiments of Compound 1. A bulb of *A. stipitatum* Regel (2.1 g fresh weight) was cleaned, sliced, and placed in 10 mL of methanol to inhibit the enzymatic activity. After 20 min of heating under reflux, the extract was filtered, the solvent was evaporated under reduced pressure, and the residue was rediluted in 1 mL of methanol. This solution was filtered through a 0.2 μm cellulose acetate filter and injected directly into the MS device.

Plant Processing for Analysis of Volatile Sulfur Compounds. Several bulbs of *A. stipitatum* Regel (53.3 g fresh weight) were cleaned and sliced. To enable the alliinase reaction, the material was placed in 400 mL of water and shaken for 30 min. Afterward, the water phase was extracted with three portions of 40 mL of ethyl acetate p.a. The combined ethyl actetate layers were evaporated at 30 °C under reduced pressure until dryness and rediluted in 5 mL of methanol. Samples were cooled down to 3 °C and analyzed immediately.

Plant Processing for Structure Elucidation of Nonvolatile Compounds. Bulbs of *A. stipitatum* Regel (105 g fresh weight) were cleaned, sliced, and instantly placed in methanol to inhibit enzymatic activity. After 20 min of heating under reflux, the pieces were homogenized and extracted under reflux for further 20 min. The extract was filtered, and the solvent was removed under reduced pressure. The obtained residue was resolved in 30 mL of OPA reagent and 300 μL of *t*-butylthiol. After an incubation time of 30 min, 500 μL of a methanolic iodacetamide dilution (1 mM) was added. The solution was filtered through a 0.45 μm cellulose acetate filter after 3 min of incubation. The solvent was removed under reduced pressure, and the residue was stored at −20 °C for further processing. Immediately before LC separation, frozen samples were rediluted in 5 mL of ammonium acetate buffer (50 μM).

HPLC Separation of OPA-Derivatized Cysteine Sulfoxides. A Waters HPLC system (600 E System controller and a Waters 991 PAD) was used for separation. LC separations were performed using a VP 250/21 Nucleodur 100-5 C18 EC column (Macherey Nagel). The column oven was set to 30 °C. An acetonitrile/50μM ammonium acetate buffer, pH 6.5, gradient with a constant flow rate of 10 mL/min was used (A, acetonitrile; B, ammonium acetate buffer): 78% B for 25 min; 78−75% B over 25 min; UV detection at 334 nm. The OPA-derivatized cysteine sulfoxide eluted at 35 min. The solvent of the collected fractions of **1a** was evaporated under reduced pressure at 30 °C. To remove traces of ammonium acetate by sublimation, the fractions were further dried under high vacuum for several hours. Afterward, the fractions were stored in the freezer at −20 °C until further analysis.

HPLC Separation of the Volatile Sulfur Compounds. The Waters HPLC system already described above was used. LC separations were performed using a VP 250/21 Nucleodur 100-5 C18 EC column (Macherey

Nagel). A methanol/water gradient with a constant flow rate of 10 mL/min was used (A, methanol; B, water): 90% B for 2 min; 90−10% B over 15 min; UV detection with a photodiode array (PDA) over a range of 200−400 nm. The solvent of the collected fractions containing **3** and **6** was removed under reduced pressure at 30 °C. Afterward, the fractions were stored in the freezer at −20 °C.

HPLC-MS Analysis of Volatile Sulfur Compounds. HPLC-ESI-MS measurements were performed on a Shimadzu LC 20 HPLC system containing an autosampler, a high-pressure mixing pump, a column oven, and an UV detector in combination with a QTrap 2000 equipped with a TurboIonspray ion source (Applied Biosystems/MDS Sciex, Toronto, Canada). The LC ESI-MS operating conditions for the analysis of **3**−**7** were as follows (positive ionization mode): scan range, 30−500 amu; source temperature, 200 °C; ion spray voltage, 5.5 kV; curtain gas, 10; declustering potential, 110 V; entrance potential, 11 V; flow rate, 0.2 mL/min (HPLC separation). Chromatographic conditions were as follows: a methanol/water gradient with a constant flow rate of 0.2 mL was used (A, methanol; B, water): 90% B for 2 min; 90−10% B over 13 min; 10−5% B over 15 min; and 5% B for 15 min. A 250/2 Nucleodur 100-5 C18 EC column (Macherey Nagel) was used for the separation.

The ESI-MS/MS operating conditions for the fragmentation of **1** and **3**−**7** were as follows: positive or negative ionization mode; scan range, 30−1000 amu; source temperature deactivated; ion spray voltage, 5.5 kV; curtain gas, 10; declustering potential, 110 V; entrance potential, 10 V; collision energy, 22−52; collision cell entrance potential, 25.42 V; collision cell exit potential, 3 V; and flow rate, 20 μL/min (direct injection).

Structure Elucidation. NMR experiments were performed on a JEOL ECA-500 spectrometer. By the aid of standard correlation experiments [correlated spectroscopy (COSY), heteronuclear multiple quantum coherence (HMQC), and heteronuclear multiple bond coherence (HMBC)] and nuclear Overhauser effect spectroscopy (NOESY) experiments, structure elucidation of compounds **1a** and **3** was performed. IR measurements were performed on Bruker Alpha-P Fourier transformation infrared (FT-IR) (Ettlingen, Germany). The HR-ESI experiment of **1a** was carried out on a Finnigan LTQ-FT hybrid mass-spectrometer. HR-ESI experiments of the compounds **3**, **4**, **6**, and **7** were performed with a Micromass Autospec (Manchester, England). The optical rotation of **1a** was measured on a Chemdata Jasco DIP-370 Digital Photometer (Zimmern, Germany) at 589 nm and 20 °C. The sample was dissolved in methanol.

Analytical Data of the Identified Compounds. Compound **1** (**Figure 1**): ESI MS/MS, m/z (relative intensity %) 70 (13), 78 (5), 88 (44), 110 (8), 112 (8), 124 (4), 128 (100), 152 (3), 159 (4), 169 (6), 198 (14), 215 (31).

Compound **1a** (**Figure 1**): ^{13}C NMR (125.77 MHz, acetonitrile-d_3): δ 30.3 (C22, C23, C24), 35.5 (C8), 49.7 (C21), 59.3 (C9), 110.6 (C19), 115.2 (C12), 120.0 (C17), 120.4 (C14), 120.9 (C15), 121.0 (C5), 122.1 (C16), 122.7 (C4), 124.5 (C13), 126.8 (C3), 130.6 (C18), 138.7 (C6), 151.7 (C2), 172.3 (C10). ^{1}H NMR (500.16 MHz, acetonitrile-d_3): δ 1.14 (s, 9, H22, H22, H22, H23, H23, H23, H24, H24, H24), 3.56 (dd, 1, J = 14.03, 10.02 Hz, H8), 3.89 (dd, 1, J = 14.03, 4.58 Hz, H8), 5.84 (dd, 1, J = 10.02, 4.58 Hz, H9), 6.88 (dt, 1, J = 0.86, 8.31 Hz, H15), 6.96 (dt, 1, J = 0.86, 8.31 Hz, H16), 7.07 (dt, 1, J = 6.30, 7.45 Hz, H5), 7.29 (dt, 1, J = 7.73, 8.02 Hz, H4), 7.43 (d, 1, J = 8.02 Hz, H3), 7.5 (d, 1, J = 8.59 Hz, H14), 7.58 (dd, 1 J = 8.59, 0.86 Hz, H17), 7.67 (s, 1, H12), 8.13 (dd, 1, J = 6.30 Hz, H6). FT-IR [ν_{max} cm^{-1}, intensity given as strong (s), medium (m) or weak (w)]: 3187 (m), 3013 (m), 2858 (m), 1694 (m), 1544 (m), 1402 (s), 1366 (s), 1269 (m), 1046 (w), 1015 (m), 923 (w), 883 (w), 833 (w), 748 (w), 658 (s), 621 (s), 491 (m), 448 (s). UV (λ_{max}, MeOH): 233, 320, 335. HR-ESI-MS, 403.1144; molecular formula $C_{20}H_{23}S_2O_3N_2$; MS calculated (M + H$^+$), 403.1145. Sodium adduct $C_{20}H_{22}S_2O_3N_2Na$: HR-ESI-MS, 425.0964; MS calculated, 425.0964; specific optical rotation [α_D^{20}], −9.2 mL g^{-1} dm^{-1} (589 nm, in methanol).

Analytical Data of Isolated Volatile Sulfur Compounds. Compound **3**: ^{1}H NMR (500.16 MHz, methanol-d_4): δ 7.40 (dt, 2, J = 1.72, 6.59 Hz, H5, H5′), 7.54 (dt, 2, J = 1.15, 8.31 Hz, H4, H4′), 7.70 (dd, 2, J = 1.72, 8.31 Hz, H3, H3′), 8.41 (dd, 2, J = 1.15, 6.59 Hz, H6, H6′). ^{13}C NMR (125.77 MHz, acetonitrile-d_3) are given in the **Table 1**. FT-IR [ν_{max} cm^{-1}, intensity given as strong (s), medium (m) or weak (w)]: 3103 (w), 2932 (m), 2861 (w), 1635 (w), 1590 (m), 1556 (w), 1519 (m), 1465 (s), 1422 (s), 1266 (m), 1249 (w), 1221 (m), 1206 (m), 1139 (m), 1083 (m), 1035 (m), 838 (s), 801 (w), 760 (s), 703 (m). UV ($\lambda_{max,}$ MeOH): 238, 264, 310. ESI MS/MS, m/z (relative intensity): 78 (100), 79 (11), 98 (16), 111 (5), 126 (29), 127 (18), 142 (18), 253 (26). HR-ESI-MS, 253.0130; molecular formula $C_{10}H_9N_2O_2S_2$. MS calculated (M + H$^+$): 253.0105. Compound **4** UV ($\lambda_{max,}$ MeOH): 238, 264, 310. ESI MS/MS, m/z (relative intensity): 66.9 (16), 78 (100), 79 (7), 98 (18), 111 (57), 126 (37), 127 (18), 237 (57). HR-ESI-MS, 237.0140; molecular formula $C_{10}H_9N_2OS_2$. MS calculated (M + H$^+$): 237.0156. Sodium adduct $C_{10}H_9N_2OS_2Na$: HR-ESI-MS, 259.0025; MS calculated, 258.9976. Compound **5** UV ($\lambda_{max,}$ MeOH): 238, 282. ESI MS/MS, m/z (relative intensity): 67 (8), 79 (3) 111 (100), 187 (8), 221 (49). Compound **6** FT-IR [ν_{max} cm^{-1}, intensity given as strong (s), medium (m) or weak (w)]: 3102 (w), 2921 (m), 1737 (m), 1679 (m), 1557 (w), 1509 (w), 1466 (s), 1422 (s), 1372 (w), 1316 (w), 1263 (w), 1245 (w), 1221 (m), 1206 (m), 1137 (w), 1080 (w), 1040 (s), 836 (m), 800 (w), 761 (m), 703 (w). UV (λ_{max}, MeOH): 238, 260, 310. ESI MS/MS, m/z (relative intensity): 78 (89), 98 (17), 111 (20), 114 (40), 125 (38), 126 (54), 127 (43), 173 (14), 174 (100). Sodium adduct $C_6H_7NOS_2Na$: HR-ESI-MS, 195.9873; MS calculated, 195.9867. Compound **7** UV (λ_{max}, MeOH): 238, 260, 310. ESI MS/MS, m/z (relative intensity): 45 (10), 61 (23), 63 (26), 78 (9), 93 (100), 109 (11), 111 (11), 112 (12), 126 (4), 142 (3) 174 (5), 220 (5). Sodium adduct $C_7H_9NOS_3Na$: HR-ESI-MS, 241.9749; MS calculated, 241.9744.

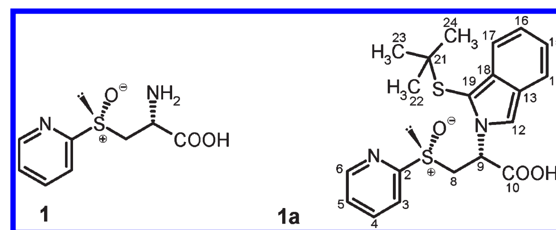

Figure 1. Structure of the newly described L-(+)-*S*-(2-pyridyl)-cysteine sulfoxide (**1**) and its OPA derivative (**1a**), which was used for structure elucidation.

Table 1. NMR Data of the Pyridyl Residue of Compound **1a** and **3**

	compound **1a** (acetonitrile-d_3) [ppm]			compound **3** (methanol-d_4) [ppm]	
no.	^{1}H	^{13}C	no.	^{1}H	^{13}C
2		151.7	2,2′		153.3
3	7.43 (d, 1, J = 8.0 Hz)	126.8	3,3′	7.70 (dd, 2, J = 1.7, 8.3 Hz)	129.2
4	7.29 (dt, 1, J = 7.7, 8.0 Hz)	122.7	4,4′	7.54 (dt, 2, J = 1.2, 8.3 Hz)	123.5
5	7.07 (dt, 1, J = 6.3, 7.5 Hz,)	121.0	5,5′	7.40 (dt, 2, J = 1.7, 6.6 Hz)	122.3
6	8.13 (dd, 1, J = 6.3 Hz)	138.7	6,6′	8.41 (dt, 2, J = 1.2, 6.6 Hz)	139.1

RESULTS AND DISCUSSION

Isolation and Structure Elucidation of the Precursor L-(+)-*S*-(2-Pyridyl)-cysteine Sulfoxide. To elucidate the structure of the proposed alliinase precursor **1**, the OPA derivatization method for amino acids was used. This strategy was chosen because direct isolation of **1** failed due to large impurities caused by various sugars. Derivatization with OPA and a mercapto-alkyl (OPA reagent) allows isolation of a nearly pure fraction of compound **1a**. MS data of **1** and **1a** emphasized a substance with two nitrogen atoms and, for **1a**, a mass difference in accordance with an OPA derivatization reaction (**1**, 214 g/mol; **1a**, 402 g/mol; mass difference, 188 amu). Furthermore, a molecular formula of $C_{20}H_{23}S_2O_3N_2$ was determined by high-resolution mass spectrometry (HR-MS) (**1a**).

Further structure elucidation was performed mainly by various NMR techniques. First, correlation experiments (HMQC, HMBC, and COSY) were used. The OPA moiety of **1a** consists

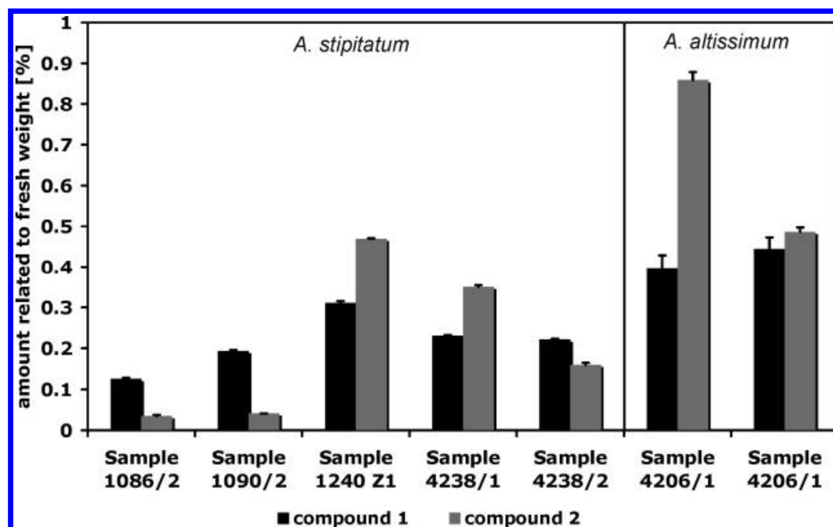

Figure 2. Amounts of cysteine sulfoxides in various samples of *A. stipitatum* Regel and *A. altissimum* Regel.

of an isoindole system and a *t*-butylthiol. These substructures exhibited characteristic signals in NMR experiments. The HMBC spectrum showed a strong correlation between the *t*-butyl alkyl protons (H22, H23, H24; δ 1.14) and the quaternary carbon atom (C21; δ 49.7). The isoindole substructure was elucidated by all three correlation experiments. COSY experiments showed specific signals for the isoindole ring. Significant here are the correlations between the protons H14 (δ 7.5), H15 (δ 6.88), H16 (δ 6.96), and H17 (δ 7.58). The HMBC correlations between the quaternary carbon atoms C13 (δ 124.5), C18 (δ 130.6), and C19 (δ 110.6) with the protons of the isoindole ring H12 (δ 7.58), H14, H15, H16, and H17 proved the expected substructure of the OPA moiety.

The cysteine structural part was identified by comparison of the NMR data with those of already published cysteine sulfoxides, like L-(+)-alliin (*20*). The signals of the protons H8 (δ 3.56, δ 3.89) matched the expected shifts in ^{1}H NMR. COSY and HMBC correlation of the protons H8 with the proton at the stereogenic center C9 (H9, δ 5.84) was obvious. The carbonic acid C10 (δ 172.3) was identified by HMBC experiments. A strong ^3J coupling of H9 and a ^4J coupling of the two hydrogens of C8 were obtained.

Finally, the sulfoxide moiety and its substitution had to be identified. No further aliphatic protons and carbons were observed in the NMR spectra. All data strongly supported a pyridyl substituent. ^{1}H NMR measurement showed four proton signals with a shift between 7.0 and 8.2 ppm. These signals showed couplings in COSY and HMBC. The couplings correlated in a clear order; it could be deduced that these protons are in positions 3 (H3 δ 7.43), 4 (H4 δ 7.29), 5 (H5 δ 7.07), and 6 (H6 δ 8.13) of the pyridyl substituent. Therefore, the sulfoxide group must be placed at position 2 of the pyridine ring.

Until now, mainly (+)-sulfoxides were found in nature. Relating to ^{1}H NMR spectra of synthetic (−)- and (+)-cysteine sulfoxides (*20*), compound **1a**, respectively, compound **1**, is the sulfur (+)-isomer. For (+)-cysteine sulfoxides, the two protons at C8 show a difference in chemical shifts between 0.2 and 0.3 ppm. The signals of these protons nearly merge to one single signal in the case of (−)-cysteine sulfoxides. For instance, the chemical shifts of the two protons at this carbon atom are 3.21 and 3.45 ppm for (+)-alliin (*20*), but 3.36 and 3.43 ppm for (−)-alliin. The precursor **1a** showed a difference of 0.33 ppm between the chemical shifts of the two protons at C8 suporting a (+)-sulfoxide. The slightly larger shift in comparison to literature data can be explained by the isoindole ring, which is located at C9.

The comparison of IR measurements of (−)- and (+)-cysteine sulfoxides underlined that compound **1a** is a (+)-isomer. The absorption band of the sulfoxide of compound **1a** shows the same profile as IR measurement of (+)-cysteine sulfoxides (*20*). A rather strong band at 1015 cm^{-1} supports the (+)-configuration, whereas the (−)-sulfoxide shows a band at 1000 cm^{-1}.

The decreased optical rotation of compound **1a** in comparison to the optical rotations of (+)-cysteine sulfoxides is caused by the OPA substituent at C9. The published specific optical rotation of L-(+)-alliin is given as $[\alpha_D{}^{20}]$ +62.8 mL g^{-1} dm^{-1} (*21*). The specific optical rotation of the derivatized precursor **1a** at $[\alpha_D{}^{20}]$ −9.2 mL g^{-1} dm^{-1} (589 nm, in methanol) is due to the larger substituent at C9.

In MS/MS experiments, compound **1** showed several characteristic fragments leading to the assumed structure. The most important fragment is 78 *m*/*z* in positive ionization mode, which indicates the pyridyl moiety. The fragment of 88 *m*/*z* in positive ionization mode is typical for cysteine derivatives. The results obtained underline the hypothesis that compound **1** is a L-(+)-cysteine sulfoxide.

Qualitative and Quantitative Analysis of *A. stipitatum* and *A. altissimum* Samples. All investigated samples of *A. stipitatum* and *A. altissimum* contained the new cysteine sulfoxide **1** and the already described methiin **2**, which could be clearly identified by HPLC-MS/MS and authentic standards (*20*). These compounds were also analyzed as their corresponding OPA derivatives. This method allows sufficient separation of cysteine sulfoxides from various amino acids, which are also present in all *Allium* samples. The amount of the new cysteine sulfoxide **1**, related to the fresh weight of bulbs, was found to be between 0.13 and 0.44%. Methiin was present in amounts of 0.03−0.86% (**Figure 2**). The examined bulbs contained **1** in amounts comparable to methiin. This suggests that numerous mixed thiosulfinates can be expected by the reaction of alliinase with these two cysteine sulfoxides (compare **Figure 3**).

High variations in the amounts of **1** and **2** can have several reasons: (i) Material was collected at various places in nature; (ii) the time of harvest was not the same for all bulbs; and (iii) it can be assumed that *A. stipitatum* and related species are taxonomically not sufficiently described. The species *A. stipitatum* can probably be divided in a number of subspecies. The area of distribution is strongly disrupted by large desert areas and mountain ranges higher as 5000 m. Leaves can show strong and dense hairs but are sometimes also nearly hairless.

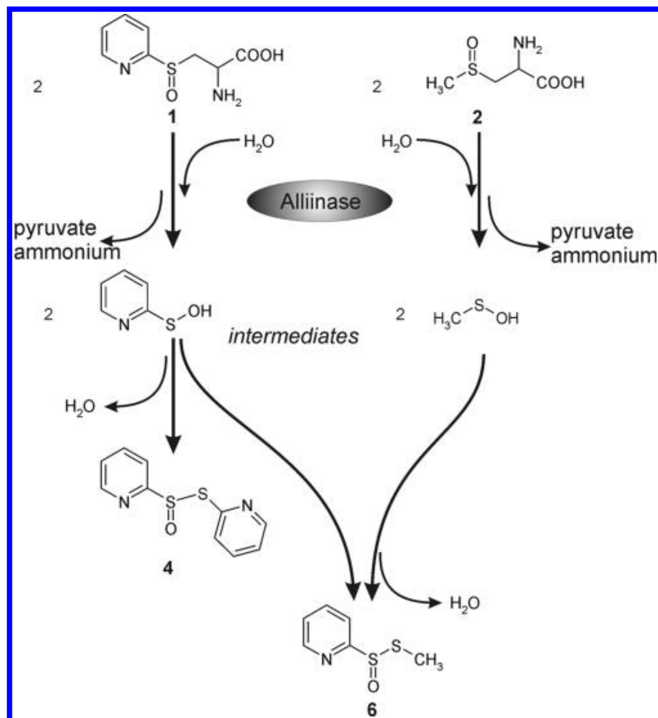

Figure 3. Alliinase-catalyzed reaction starting with L-(+)-*S*-(2-pyridyl)-cysteine sulfoxide (**1**) and methiin (**2**) as precursors.

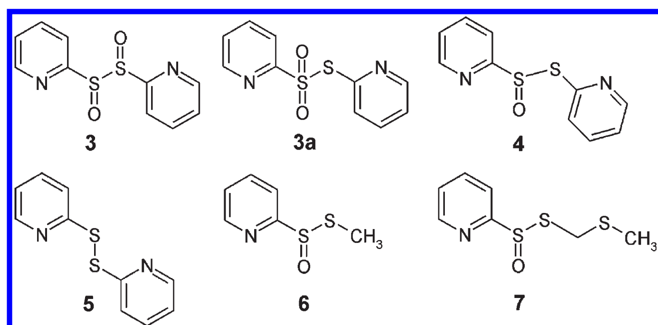

Figure 4. Structures of volatile compounds found after alliinase reaction. Substance **3a** was synthesized according to Barton and Ramesh (*22*) to perform structure elucidation of **3**.

Analysis of Volatile Sulfur Compounds. According to the alliinase activity of *A. stipitatum* (*17*), cysteine sulfoxides should first react to thiosulfinates as primary volatile compounds. However, the analysis of the formed metabolites was crucial, because obtained yields were rather low. To exclude further reaction of formed thiosulfinates, the extract was processed and analyzed rapidly after alliinase reaction. Under these conditions, the formation of volatile compounds should follow the pathway described in **Figure 3**.

Identified compounds are given in **Figure 4**. The pure fractions containing **3** and **6** were stored at −20 °C to minimize side reactions. Structure elucidation was performed by ^{1}H NMR, FT-IR, HPLC MS/MS, and MS/MS fragmentation. According to the alliinase reaction depicted in **Figure 3**, compounds **4** and **6** were expected. Beside these substances, three related volatile compounds (**3**, **5**, and **7**) were identified by HPLC-MS/MS. HR-ESI-MS data of **3**, **4**, **6**, and **7** were gathered and showed good correlation with the proposed structures. Typical MS/MS fragments found by MS/MS fragmentation are given in **Figure 5**. Compound **5** is known as “aldrithiol-2”, which can be obtained from Sigma-Aldrich (Munich, Germany). Retention time as well

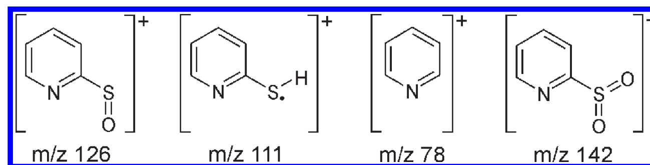

Figure 5. Main fragments of pyridyl compounds found in MS/MS experiments (positive ionization mode).

as MS fragmentation of **5** and aldrithiol-2 were found to be identical.

The HPLC-MS/MS measurements showed correlations between the retention times and the polarity of the proposed structures. Peaks related to compounds **3**−**5** showed MS signals correlated to retention times as follows (RP column): **3**, *m*/*z* 253 $[M+H]^+$, 16.4 min; **4**, *m*/*z* 237 $[M+H]^+$, 22.4 min; and **5**, *m*/*z* 221 $[M+H]^+$, 27.5 min. This change in retention time correlates with the amount of oxygen in the different compounds (**3**, two oxygens; **4**, one oxygen; and **5**, no oxygen). Compound **6** eluted at 19.2 min, and compound **7** eluted at 23.12 min.

MS/MS experiments of compounds **3**, **4**, **6**, and **7** gave a *m*/*z* 126 $[M]^+$ fragment, which indicates the pyridyl sulfoxide moiety, and a *m*/*z* 78 $[M]^+$ fragment, which is characteristic for the pyridyl moiety (**Figure 5**). The fragment at *m*/*z* 111 $[M + H]^+$ for compounds **3**−**7** emphasizes the presence of a pyridyl disulfide substructure. This fragment might be formed by a homolytic cleavage of the disulfide bridge. The presence of *m*/*z* 126 $[M]^+$ suggests that compounds **6** and **7** were products of **1** and **2** with a thiosulfinate moiety, which is in full accordance with the alliinase reaction theory. Due to the fragment *m*/*z* 126, this oxygen must be located at the sulfur next to the pyridyl substituent. Furthermore, the intensity of *m*/*z* 126 $[M]^+$ is much higher as the intensity of *m*/*z* 111 $[M+H]^+$.

For compound **3**, a strong signal at *m*/*z* 142 $[M]^+$ was found resulting from a homolytic cleavage between the two sulfoxy functionalities. To prove the suggested structure of **3**, compound **3a** was synthesized according to Barton and Ramesh (*22*) and subjected to MS/MS fragmentation experiment. The fragmentation pattern was found to be completely different from **3**.

Compounds **3** and **6** were separated by HPLC in a semipreparative scale and examined by FT-IR measurements. Compound **6** showed the typical absorption bands of a pyridyl moiety at 702, 762, 836, 1206, 1220, 1249, 1266, 1422, and 1466 cm^{-1}. Beside these pyridyl bands, the typical band of the expected thiosulfinate was observed with a strong intensity at 1040 cm^{-1}. Compound **3** also showed the typical pyridyl bands. This substance was redissolved in methanol-d_4 and further analyzed by ^{1}H NMR techniques. The four expected proton signals of the two identical pyridyl moiety occurred at δ 7.70 (H3, H3′), 7.54 (H4, H4′), 7.40 (H5, H5′), and 8.41 (H6, H6′). This suggests that **3** is a disulfoxide.

As alternative structure, compound **3a** is thinkable. The synthetic reference material of **3a** was also subjected to NMR experiments showing eight different proton signals in ^{1}H NMR (between δ 7.4 and 8.7 instead of four proton signals for **3**). H^2 COSY measurements of **3a** showed the expected correlation of the proton signals. Additionally, there were differences in the IR spectrum between **3** and **3a**. The found ^{1}H NMR between **1a** and **3** is rather similar, strongly supporting the supposed structure of **3**.

In MS/MS experiments, the main fragments of compound **3** were *m*/*z* 142 $[M]^+$, *m*/*z* 126 $[M]^+$, and *m*/*z* 78 $[M]^+$. The *m*/*z* 142 $[M]^+$ fragment is formed due to an intramolecular shift of the oxygen during the fragmentation. However, the structure of **3** does not fit with the “classical” alliinase theory (disulfoxide

instead of a thiosulfinate). In previous experiments of our group, it was found that alliinase isolated from *Melanocrommyum* species has a different substrate specificity as alliinase from *A. sativum* and *A. cepa* (*8*). As a remarkable feature of the *Melanocrommyum* alliinase, an oxidase side activity was found. Therefore, the formation of the second sulfoxide group of **3** instead of a thiosulfinate can be a direct effect of alliinase activity. However, this oxidase activity needs further investigation.

Special attention was paid to the question whether the found oxygen atoms are attached to the sulfur atom or to the nitrogen of the pyridine ring. The alliinase pathway only allows attachment to the sulfur. According to O'Donnell et al. (*9*), one antimicrobial component of *A. stipitatum* is a 2,2′-dithio-bis-pyridine-*N*-oxide. The ESI-MS measurements of this substance showed a mass signal of *m*/*z* 220 $[M]^+$ (*10*). This fragment had a relative intensity of 20% and is formed by splitting of the two pyridine N−O bonds. This fragment did not occur in the MS/MS measurements of compound **3**. Furthermore, in the MS spectrum of 2,2′-dithio-bis-pyridine-*N*-oxide, a fragment at *m*/*z* 110 $[M]^+$ showed a high relative intensity of 84% (*10*). This is formed by the reduction of the nitrogens and a homolytic cleavage of the disulfide bond. In contrast, the main fragments of compound **3** were *m*/*z* 142 $[M]^+$, *m*/*z* 126 $[M]^+$, and *m*/*z* 78 $[M]^+$.

Conversion of compound **4** into a dipyridine-di-*N*-oxide is rather unlikely as a direct product of alliinase activity. The absence of a *N*-oxide is further supported by comparison of NMR data. The ^{1}H and ^{13}C NMR data of compound **1a** (precursor) and compound **3** (product) are also very similar (**Table 1**). However, formation of a pyridine-*N*-oxide would result in significant shifts in the proton NMR spectra of 3, especially at position 3 and 6 of the pyridine ring. A migration of the oxygen atom from sulfur to nitrogen is also theoretically possible. However, the dissociation enthalpy of the N−O bond of a pyridine-*N*-oxide is given as 301.7 ± 2.8 kJ mol^{-1} at 298.15 K (*18*). The dissociation enthalpy of sulfoxides is listed as 420 kJ mol^{-1} at 298.15 K (*19*). Under strict temperature and time control as applied in the here-described experiments, an intramolecular shift of the oxygen is very unlikely. Furthermore, the proposed structure of **3** was also supported by the radiolysis of 2-thiopyridine derivatives (*23*). Sulfoxides instead of *N*-oxides were observed as reaction products.

In the MS/MS measurements of compound **5**, a different fragmentation pattern in comparison to **3**, **4**, **6**, and **7** was observed. The high amount of the fragment at *m*/*z* 111 $[M + H]^+$ and the absence of fragment *m*/*z* 78 $[M+H]^+$ emphasize the presence of a disulfide bond instead of a thiosulfinate or a disulfoxide functionality. The found fragmentation pattern was identical with this reference material (aldrithiol-2).

The main fragments of compound **7** were *m*/*z* 174 $[M + H]^+$ and *m*/*z* 93 $[M]^+$. The *m*/*z* 174 $[M+H]^+$ fragment is identical with the positively charged compound **6**. The fragment of *m*/*z* 93 showed the highest intensity and is formed by the cleavage of the disulfide bond. These findings strongly support that compound **7** is homologous to **6** (one additional CH_2−S group).

UV spectra were obtained for all compounds during the HPLC separations by a PDA detector. The spectra of **3** and **4** showed three UV maxima at 238, 264, and 310 nm. The maximum at 310 nm had a lower intensity for compound **4**. Compound **5** only showed two UV maxima at 238 and 282 nm. The missing of the UV minor maxima at 310 indicates the absence of the sulfoxide functionality. The UV spectra of compounds **6** and **7** showed similarities to the spectra of compounds **3** and **4**. Three maxima at 238, 260, and 310 nm were found.

The relative amounts of the volatile sulfur compounds were estimated by the area under the curve of the UV signals at 254 nm. The total area under the curve of the intensities of the UV signals of compounds **3**−**7** was set to 100%. Compound **7** showed the largest relative amount (39.2%). The other compounds had amounts as follows: **3** (24.4%), **4** (4.6%), **5** (0.8%), and **6** (30.5%). Expected volatile compounds consisting of two methyl-sulfur moieties could not be safely determined by LC-MS experiments, probably due to low concentrations. It can be assumed that alliinase products of methiin nearly completely react to compounds **6** and **7**, which are the leading substances of the volatile fraction.

Conclusions. A new class of cysteine sulfoxides was isolated from *A. stipitatum*, which is also present in closely related species. This probably allows chemo-taxonomical classification of this section inside the subgenus *Melanocrommyum*. Still, further samples have to be analyzed. Because of high variations in the amount of cysteine sulfoxides, it can be expected that this genus has to be divided into different subgenera.

A. stipitatum is widely used as a spicy vegetable and as a traditional medicine. However, no information is available about possible toxicity of the pyridine derivatives. The observed strong antibiotic effects are an interesting observation, but it can be assumed that the sulfur compounds do also show further bioactivities. This is of importance, because bulbs of *A. stipitatum* are eaten by several million people.

Only some of the pyridine compounds of *A. stipitatum* could be elucidated. Analogously to *A. sativum* and *A. cepa*, it can be expected that different postharvest treatments of the crude bulb material will lead to different patterns of pyridine sulfur compounds. The proposed enzymatic cleavage of the L-(+)-*S*-(2-pyridyl)-cysteine sulfoxide leads to thiosulfinates like **4** and **6**. The alliinase theory only allows thiosulfinates as primary enzymatic products, also mixed thiosulfinates. The presence of **3**, **5**, and **7** is somewhat surprising and can be explained by secondary oxidation or reduction reactions. As described previously, oxidation reactions can be also directly catalyzed by *Melanocrommyum*-alliinase. Volatile compounds with a R−SO−SO−R structure are already described in literature (*3*). Normally, these compounds are intermediate products. The relatively high amount in the samples of our investigation is a surprising observation. Compound **7** is probably caused by fast reactions of two molecules of methylsulfenic acid with the 2-pyridinesulfenic acid (see **Figure 3**). The primarily formed thiosulfinates are highly reactive and are probably subject to secondary and tertiary modifications. It is unclear if further enzymatic steps are involved in this procedure. Nevertheless, the results strongly emphasize that *N*-oxides were not formed during or directly after alliinase reaction. The already described *N*-oxides are probably caused by further fermentation processes. Because of the complicated taxonomy of *A. stipitatum*, it can also be assumed that investigations described in ref *9* were performed with a different species.

ABBREVIATIONS USED

OPA, *o*-phthaldialdehyde; COSY, correlated spectroscopy; HMQC, heteronuclear multiple quantum coherence; HMBC, heteronuclear multiple bond coherence; NOESY, nuclear Overhauser effect spectroscopy; MS, mass spectrometry; ESI, electrospray ionization; HR-MS, high-resolution mass spectrometry; HPLC, high-performance liquid chromatography; FT-IR, Fourier transformation infrared; PDA, photodiode array.

SAFETY

The smell and toxicity of the 2-methylpropanethiol (*t*-butylthiol) insist that the preparation of the OPA derivatization reagent be performed under a fume hood.

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Supporting Information Available: FT-IR of compounds **1a**, **3**, **3a**, and **6**; MS/MS of compounds **3** and **3a**; MS of compound **5**; and ^{1}H NMR of compounds **3** and **3a**. This material is available free of charge via the Internet at http://pubs.acs.org.

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