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Allium Discoloration: The Precursor and Formation of the Red Pigment in Giant Onion (*Allium giganteum* Regel) and Some Other Subgenus *Melanocrommyum* Species

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Supporting Information

ABSTRACT: The precursor of the orange-red pigment formed upon wounding the bulbs of *Allium giganteum* (*Allium* subg. *Melanocrommyum*) was isolated and shown to be *S*-(2-pyrrolyl)cysteine *S*-oxide. In addition, two other pyrrolylsulfinyl derivatives were found in an extract from the bulbs, namely, 3-(2-pyrrolylsulfinyl)lactic acid and *S*-(3-pyrrolyl)cysteine *S*-oxide. Contrary to a previous report, the latter compound was shown not to serve as the precursor of the pigment, being in fact only an artifact formed during isolation. The formation of pyrrolyl-containing compounds following disruption of *A. giganteum* bulbs was studied by a combination of LC−MS, LC−NMR and DART-MS. It was found that *S*-(2-pyrrolyl)cysteine *S*-oxide is cleaved by a C−S lyase (alliinase) to yield 2-pyrrolesulfenic acid. Two molecules of the latter compound give rise to highly reactive *S*-(2-pyrrolyl) 2-pyrrolethiosulfinate which in turn converts into red 2,2′-epidithio-3,3′-dipyrrole (dipyrrolo[2,3-*d*:2′,3′-*e*]-1,2-dithiin). Several other pyrrolyl-containing compounds were detected in *A. giganteum* for the first time, including *S*-methyl 2-pyrrolethiosulfinate, *S*-(2-pyrrolyl) methanethiosulfinate, di(2-pyrrolyl) disulfide, and *S*-(2-pyrrolyl) 2-pyrrolethiosulfonate. It can be concluded that the formation of the orange-red pigment in *Allium* subg. *Melanocrommyum* species, despite sharing several analogous features, is of a different nature than the pink discoloration of onion (*A. cepa*).

KEYWORDS: *S*-(2-pyrrolyl)cysteine *S*-oxide, *S*-(3-pyrrolyl)cysteine *S*-oxide, giant onion, *Allium giganteum*, *Melanocrommyum*, discoloration, thiosulfinate, pigment, LC−NMR, DART-MS, sulfenic acid, non-protein amino acid

INTRODUCTION

The genus *Allium* L. (Alliaceae) comprises more than 800 different species growing mostly in the Northern hemisphere. Due to its large diversity, the genus is currently divided into 15 subgenera, with *Melanocrommyum* being one of the largest groups. The subgenus *Melanocrommyum* (Webb & Berthel.) Rouy comprises about 160 mostly perennial species native to arid regions of the Mediterranean, the Near and Middle East, northwestern China, Pakistan and Central Asia.[1,2] Many of these plants are frequently consumed (e.g., *A. stipitatum* or *A. rosenbachianum*) or used in traditional medicine (e.g., *A. suworowii*, *A. motor* or *A. hissaricum*) to treat a variety of disorders.[3] Thanks to their attractive and long-lasting inflorescences, many subgenus *Melanocrommyum* species are popular ornamental plants and some are commercially cultivated for landscaping purposes.

A. giganteum Regel belongs to the tallest *Allium* species, hence its common name "giant onion". It produces sturdy stalks up to 160 cm tall, each bearing a fireworks-like cluster of star-shaped, purple flowers with a diameter of about 20 cm. This bulbous plant is native to the dry steppes of Eastern and Central Asia. Giant onion is known for its ability to produce remarkable amounts of an intensely orange-red exudate upon wounding the bulbs or leaves (Figure 1). Reportedly, this pigment is locally used for dyeing of clothes.[4]

Figure 1. Color changes of *A. giganteum* bulb induced by cutting.

We were attracted to the orange-red pigment of giant onion due to our long-term interest in colored compounds formed in alliaceous plants following tissue disruption. For example, the color of garlic (*Allium sativum*) often turns green-blue during pickling,[5] whereas undesirable pink discoloration can develop during industrial or culinary processing of onion (*Allium cepa*).[6] Although the discoloration of garlic and onion has been extensively studied, this economically important phenomenon is still

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not completely understood. It has been found that the discoloration of both onion and garlic is of a very similar nature, with *S*-(*E*)-(1-propenyl)cysteine *S*-oxide (isoalliin) being the primary precursor.[7−10] Isoalliin, together with other *S*-alk(en)ylcysteine *S*-oxides (mainly methiin and alliin), is enzymatically cleaved upon disruption of the tissue, yielding 1-propenyl-containing thiosulfinates [(*E*)-CH_3CH═CHS(O)SR and (*E*/*Z*)-CH_3CH═CHSS(O)R]. These thiosulfinates subsequently react with amino compounds to give a variety of *N*-substituted derivatives of 3,4-dimethylpyrrole[7−12] (Figure 2).

The formation of the pigment in giant onion is noticeably faster compared to that in common onion. Whereas the orange-red exudate in *A. giganteum* is formed nearly instantly after tissue disruption, the formation of pink discoloration in onion takes much longer (typically several hours at room temperature). Joslyn and Peterson[6] were the first to study the red compounds formed in *A. giganteum*. They noticed a close similarity of UV−vis spectra of the pigments formed upon tissue disruption of *A. giganteum* and common onion. Jedelská et al.[4] recently reported the pigment precursor in giant onion to be *S*-(3-pyrrolyl)cysteine *S*-oxide (3-PyrrCSO). It was proposed that this amino acid is cleaved by alliinase to yield a red pyrrole derivative 3,3′-epidithio-2,2′-dipyrrole (alternative name [2,3-*c*:2′,3′-*f*]-1,2-dithiin).

In this paper, we report the results of our studies of the orange-red pigment formed in *A. giganteum* and several other subgenus *Melanocrommyum* species upon tissue disruption. Besides other novel findings, we present evidence that the structure of the amino acid precursor of this pigment is different from that reported previously by Jedelská et al.[4]

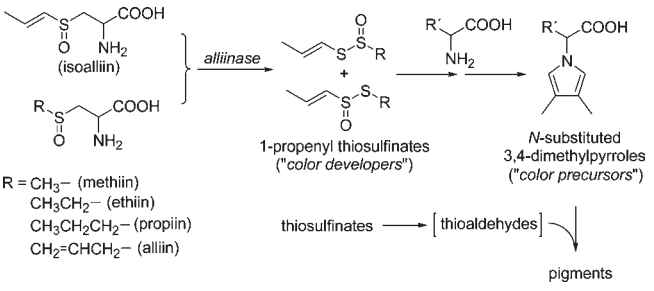

Figure 2. Formation of colored compounds during processing of garlic and onion.

RESULTS AND DISCUSSION

An amino acid-containing fraction from the bulbs of *A. giganteum* was obtained by extraction with acidified aqueous methanol ($MeOH/H_2O$/HCl 90/9/1, v/v/v) and subsequent treatment by cation-exchange chromatography. C-8 HPLC analysis of the fraction revealed the presence of several compounds exhibiting significant absorption in the region of 240−280 nm. Three of these compounds (**1**−**3**) were subsequently isolated and fully characterized by spectroscopic methods.

The major isolated compound (**1**) was found to be both Ehrlich-positive and ninhydrin-positive. Its ^{13}C NMR spectroscopic data indicated the presence of four aromatic carbons (δ 110.0, 115.9, 124.6, and 126.4 ppm), together with one carboxylic (δ 175.1 ppm) and two sp^3-hybridized (δ 51.5 and 55.2 ppm) carbon atoms. Further NMR experiments (including COSY, HETCOR, DEPT, and HMBC) revealed the presence of two isolated structural subunits: (i) a monosubstituted pyrrolyl moiety and (ii) a $-CH_2CH(X)COOH$ chain (Table 1). The IR spectrum contained a very strong absorption band at 995 cm^{-1} (−S═O) and the ESI-TOF HRMS exhibited $[M + H]^+$ of 203.0485 (calcd for $C_7H_{11}N_2O_3S$ 203.0485), indicating that the compound is an *S*-pyrrolyl substituted cysteine *S*-oxide. The most difficult task in the structure elucidation of **1** appeared to be establishing the site of pyrrole substitution by the cysteinyl moiety. Due to mutual coupling of all three pyrrolyl −CH═ hydrogens and comparable magnitudes of $^2J(^{13}C-^1H)$ and $^3J(^{13}C-^1H)$ coupling constants, COSY, HMBC, DEPT or NOESY NMR experiments could not provide a clear proof regarding the site of pyrrole substitution. Comparison of the NMR data of **1** with those of various 2- and 3-sulfinyl pyrrole derivatives reported in the literature[13−16] indicated substitution at position 2. For example, the signals of the pyrrolyl −CH═ hydrogens in 2-sulfinyl derivatives typically appear as three distinct doublets of doublets ($J_{3,4}$, $J_{3,5}$ and $J_{4,5}$ of 3.8, 1.5, and 2.8 Hz, respectively). On the other hand, the pyrrolyl hydrogens in 3-sulfinyl derivatives usually exhibit one triplet and two

Table 1. ^1H and ^{13}C NMR Data of Compounds Isolated in This Study (1−3) and the Compound Reported in Ref 4

	chemical shifts (ppm), signal intensity, multiplicity, coupling constants (Hz)			
	1	**2**	**3**	**2′**
H-2	3.86 (1H, dd, 8.2/5.2)	3.90 (1H, dd, 8.2/4.8)	4.23 (1H, dd, 10.5/3.0)	4.23 (1H, dd, 7.8/6.7)
H-3a	3.55 (1H, dd, 5.2/13.8)	3.48 (1H, dd, 4.8/13.9)	3.07 (1H, dd, 10.5,13.3)	3.64 (1H, dd, 6.7/5.7)
H-3b	3.63 (1H, dd, 13.8/8.2)	3.56 (1H, dd, 13.9/8.2)	3.69 (1H, dd, 13.3/3.0)	3.83 (1H, dd, 7.8/5.7)
H-2′		7.41 (1H, *t*, 1.8)		7.22 (1H, dd, 1.0/3.0)[a]
H-3′	6.85 (1H, dd, 1.5/3.8)		6.79 (1H, dd, 1.5/3.8)	
H-4′	6.27 (1H, dd, 2.7/3.8)	6.58 (1H, dd, 1.7/3.1)	6.24 (1H, dd, 2.7/3.8)	6.35 (1H, dd, 3.0/3.9)
H-5′	7.15 (1H, dd, 1.5/2.7)	6.97 (1H, dd, 1.9/3.1)	7.10 (1H, dd, 1.5/2.7)	6.92 (1H, dd, 1.0/3.9)[b]
C-1	175.1	171.9	178.3	174.3
C-2	51.5	51.3	67.1	51.3
C-3	55.2	53.8	57.9	53.9
C-2′	124.6	123.9	125.4	126.5[c]
C-3′	115.9	119.1	115.1	124.7[d]
C-4′	110.0	104.7	109.8	110.3
C-5′	126.4	122.4	125.8	115.9[e]

[a] Correct assignment is H-5′. [b] Correct assignment is H-3′. [c] Correct assignment is C-5′. [d] Correct assignment is C-2′. [e] Correct assignment is C-3′.

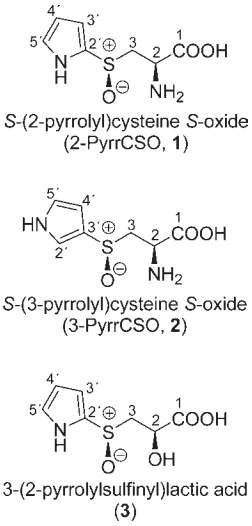

Figure 3. Structures of pyrrolyl-containing compounds isolated from *Allium giganteum* bulbs.

doublets of doublets ($J_{2,4}$, $J_{2,5}$ and $J_{4,5}$ of 1.7−1.8, 2.0−2.1 and 3.1−3.2 Hz, respectively). The signals in **1** appeared as three nicely separated doublets of doublets (J = 3.8, 1.5, and 2.7 Hz), indicating substitution at position 2. Foolproof evidence that the pyrrolyl moiety is indeed substituted at position 2 was obtained by a 1,1-ADEQUATE NMR experiment (see Supporting Information).[17]

CD and ^{1}H NMR spectroscopy together with polarimetry were used to determine the absolute configuration around the two chiral centers of **1** (around the sulfur and the α-carbon). The CD spectrum of the amino acid showed a positive maximum at 251 nm (see Supporting Information). The ^{1}H NMR spectrum of **1** contained a characteristic ABX splitting pattern for the two $-S(O)CH_2CH(NH_2)^-$ methylene protons which appeared as two distinct doublets of doublets (J_{AX} = 5.2 Hz and J_{BX} = 8.2 Hz) centered at δ 3.55 and δ 3.63 ppm, respectively. These NMR data suggested that both the amino group and the sulfoxide oxygen are on the same face of the molecule.[18,19] Furthermore, the optical rotation of the compound was found to be $[\alpha]_D^{22}$ +36.2°, indicating that the spatial arrangement about the sulfoxide group is analogous to that of other dextrorotary *S*-substituted cysteine *S*-oxides (e.g., alliin). Thus, the structure of the isolated amino acid could be unambiguously determined as (R_S,R_C)-*S*-(2-pyrrolyl)cysteine *S*-oxide (2-PyrrCSO, **1**) (Figure 3).

The second isolated compound (**2**) gave positive tests with both Ehrlich's reagent and ninhydrin, which suggested that it is also a pyrrolyl-containing amino acid. The compound exhibited very similar ESI-MS fragmentation patterns to those of **1** in both negative and positive modes (see Supporting Information), and the ESI-HRMS data showed that **1** and **2** have the same elemental composition ($C_7H_{10}N_2O_3S$). ^{1}H and ^{13}C NMR spectra of **1** and **2** differed only slightly, confirming a very close structural similarity of these two compounds (Table 1). These findings in combination with further NMR experiments (including COSY, HETCOR, HMBC, HSQC) and IR data revealed that **2** is also an *S*-pyrrolyl substituted cysteine *S*-oxide. The 1,1-ADEQUATE NMR spectrum of **2** provided fool-proof evidence that the pyrrole moiety is substituted at position 3 (see Supporting Information).[17] Furthermore, the signals corresponding to the three pyrrolyl −CH= hydrogens appeared as one triplet (H-2′) and two doublets of doublets (H-4′ and H-5′), which is fully in agreement with literature data reported for 3-sulfinyl pyrrole derivatives.[13,14]

The absolute configuration around the sulfur and the α-carbon in **2** was deducted from the sign of optical rotation and ^{1}H NMR and CD spectra. The ^{1}H NMR spectrum contained two nicely separated doublets of doublets (δ 3.48 and δ 3.56 ppm, with J_{AX} = 4.8 Hz and J_{BX} = 8.2 Hz, respectively), belonging to the two $-S(O)CH_2CH(NH_2)-$ methylene protons. Furthermore, the compound was found to be dextrorotary ($[\alpha]_D^{22}$ +45.7°) and exhibited the same sign of the Cotton effect as **1**. All these data indicated that both the amino group and the sulfoxide oxygen are on the same face of the molecule and that the absolute configuration around the sulfur is *R*. Thus, the structure of the compound could be established as (R_S,R_C)-*S*-(3-pyrrolyl)cysteine *S*-oxide (3-PyrrCSO, **2**) (Figure 3).

It has been reported, however, that 2-sulfinylpyrroles can readily isomerize to the corresponding 3-sulfinyl derivatives in acidic solutions.[13−16] Because acidified aqueous methanol was used for extraction, we decided to prove whether 3-PyrrCSO is a component genuinely present in the intact bulbs or it is only an artifact formed from 2-PyrrCSO during isolation. Pure **1** was thus subjected to the same treatment (including extraction with acidified methanol) as were the bulbs during the isolation procedure. It was found that 2-PyrrCSO extensively converted into 3-PyrrCSO when treated with cold acidified methanol (aprox. 50% conversion after 4 h, with no other side products detectable by HPLC). It is noteworthy that this isomerization proceeded with complete retention of the absolute configuration around the sulfur. To confirm the absence of 3-PyrrCSO in intact bulbs of *A. giganteum*, another extract was prepared using nonacidified aqueous methanol ($MeOH/H_2O$ 90/10, v/v). Indeed, 3-PyrrCSO was not detected in this extract, confirming that this compound is only an artifact not naturally present in the intact bulbs.

The presence of 3-PyrrCSO (**2**) in *A. giganteum* was recently reported by Jedelská et al.,[4] who claimed that this compound is the precursor of the red pigment formed upon cutting the bulbs. In the present study, however, we have shown that 3-PyrrCSO is absent in the intact bulbs and, as will be described later, alliinase-mediated decomposition of this amino acid does not yield any red compounds. Careful evaluation of the analytical data given by Jedelská et al. for the compound they isolated (**2′**) revealed several major discrepancies with our findings. As summarized in Table 1, the ^{13}C NMR shifts of **2′** are nearly identical to those of **1**. Furthermore, the signals of the pyrrolyl −CH= hydrogens in **2′** appeared as three doublets of doublets, with the chemical shifts and coupling constants corresponding to the 2-substituted derivative (**1**). Jedelská et al. reported that they deducted the structure of **2′** mainly from the HMBC NMR data. According to our experience, however, the HMBC spectrum could not provide an affirmative proof of the site of pyrrole substitution due to many comparable long-range correlations. Furthermore, we also found that the UV spectra of 2-PyrrCSO and 3-PyrrCSO differ quite considerably (perhaps due to the hydrogen bonding between the pyrrolyl −NH− hydrogen and the sulfoxide oxygen). Whereas 2-PyrrCSO exhibits UV absorption maxima at 219 and 250 nm, those of 3-PyrrCSO are located at 202 and 232 nm (see Supporting Information). Jedelská et al. reported that the UV spectrum of the compound they isolated (**2′**) exhibited maxima at 220 and 250 nm, which are values nearly identical to those we observed for 2-PyrrCSO. Thus, we believe that we gathered enough evidence to conclude that the compound isolated by Jedelská et al. (**2′**) was in fact *S*-(2-pyrrolyl)cysteine *S*-oxide (**1**).

The NMR spectra of the third compound (**3**) isolated from the bulbs were very similar to those of **1**, revealing the presence of both a monosubstituted pyrrolyl moiety and a $-CH_2CH(X)COOH$ chain (Table 1). The most obvious difference between the ^{13}C NMR spectra of **1** and **3** was observed in the chemical shifts of the C-2 carbons (δ 51.5 and 67.1 ppm, respectively), indicating that the substituent $-X$ in **3** is a more electron-withdrawing group than $-NH_2$. The compound gave a positive test with the Ehrlich's reagent, while it did not react with ninhydrin, showing that **3** probably contains a pyrrolyl moiety but it is not an amino acid. The IR spectrum of **3** contained a very strong absorption band at 987 cm^{-1} ($-S{=}O$) and the ESI-TOF HRMS exhibited $[M - H]^-$ of 202.0182 (calcd for $C_7H_8NO_4S$ 202.0180). The chemical shifts, splitting patterns (three distinct doublets of doublets) and coupling constants of the pyrrolyl $-CH{=}$ hydrogens clearly showed that the pyrrole moiety is substituted at position 2 (Table 1). Based on the aforementioned spectral data, we could conclude that **3** is 3-(2-pyrrolylsulfinyl)lactic acid. The UV and CD spectra of **3** were nearly identical with those of **1**, indicating that both compounds have the same absolute configuration around the two chiral centers, i.e. R_SR_C. Thus, the structure of **3** could be assigned as (R_S,R_C)-3-(2-pyrrolylsulfinyl)lactic acid (Figure 3).

The isolation of another pyrrolyl derivative of lactic acid, (R_S, R_C)-2-(3-pyrrolylsulfinyl)lactic acid (**3′**), from *A. giganteum* was reported by Jedelská et al.[4] According to the NMR data given, it can be assumed that the compound was rather the corresponding 2-pyrrolylsulfinyl derivative, i.e. 3-(2-pyrrolylsulfinyl)lactic acid. It was proposed that **3′** is not present in the intact tissue, but it is generated by the reaction of the enzymatically formed pyrrolesulfenic acid with pyruvic acid following tissue disruption. However, we consider this proposal highly questionable, because four different stereoisomers of **3′** would be formed by this hypothetical reaction but only one was found. Besides, we could not confirm the presence of **3′** in any of our samples or model mixtures although we specifically searched for components with M_r 203 other than **3** by LC−MS.

GC−MS and HPLC analysis of the amino acid fraction isolated from *A. giganteum* bulbs also revealed the presence of another *S*-substituted cysteine derivative, namely, (S_S,R_C)-*S*-methylcysteine *S*-oxide (methiin, MCSO, **4**). This well-known amino acid is commonly present in all alliaceous species[19−21] and its finding in *A. giganteum* is in accordance with the previous report.[4] The relative ratio of **1**/**4** was found to be 79/21 in the bulbs of *A. giganteum* we analyzed. None of the other cysteine derivatives monitored in this study was detected in the amino acid fraction from the bulbs.

In theory, the two cysteine derivatives present in the intact bulbs, 2-PyrrCSO (**1**) and MCSO (**4**), should give rise to four thiosulfinates (two symmetrical and two unsymmetrical ones) under the catalysis by alliinase (Figure 4). In order to identify the products of alliinase-mediated decomposition of **1** and **4**, a diethyl ether extract of homogenized bulbs of *A. giganteum* was prepared. The expected presence of the four thiosulfinates (**6**−**9**) in the extract was immediately monitored by LC−MS. To our surprise, none of the 2-pyrrolyl-containing thiosulfinates (**6**−**8**) were detected, despite the abundant presence of their precursor (2-PyrrCSO, **1**) in the bulbs. Thus, we attempted to generate these three thiosulfinates in model systems consisting of **1**, **4** and partially purified alliinase (EC 4.4.1.4) from either *A. giganteum* or onion. The color of all model mixtures containing 2-PyrrCSO turned orange immediately after mixing the components, indicating that an

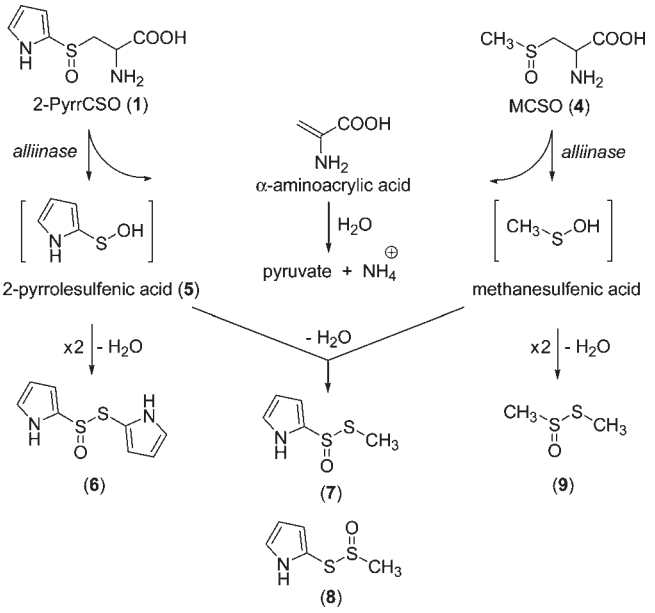

Figure 4. Alliinase-mediated formation of compounds in *Allium giganteum*.

enzymatically catalyzed reaction took place. Interestingly, there was no obvious visual difference observed when either the alliinase from *A. giganteum* or onion was employed. However, no components with the expected molecular weights (M_r 212 for **6** and M_r 161 for **7**/**8**) were detected by LC−MS in any model mixture. These results indicated that compounds **6**−**8**, if formed, are very short-living species.

To confirm the expected formation of thiosulfinates **6**−**8** in *A. giganteum*, we decided to follow the alliinase-mediated conversion of 2-PyrrCSO and MCSO by direct analysis in real time mass spectrometry (DART-MS). This exceptionally mild analytical technique allows one to observe formation of compounds of only a fleeting existence without the necessity for prior treatment, simply by momentarily holding the sample in the DART gas stream.[19,22] A bulb of *A. giganteum* was punctured by a sampling capillary, which was immediately (within 2−3 s after tissue disruption) inserted in the source region. Indeed, signals corresponding to pyrrolyl-containing thiosulfinates **6**−**8** were detected by PI-DART-HRMS (Table 2). Although DART is not able to distinguish various isomers (unless additional MS/MS measurements are performed), it is reasonable to assume that both regiomers **7**/**8** were formed. Furthermore, the presence of 2-pyrrolesulfenic acid (**5**) was detected by NI-DART-HRMS. The formation of all these compounds (**5**−**9**) was also observed in various model mixtures consisting of 2-PyrrCSO, MCSO and alliinase. To the best of our knowledge, sulfenic acid **5** and thiosulfinates **6**−**8** are novel compounds, not previously reported in the literature.

On the other hand, 3-PyrrCSO (**2**), the *S*-pyrrolylcysteine derivative not naturally occurring in *A. giganteum*, yielded a single compound (**10**) upon mixing with alliinase. This enzymatically formed product was shown to exhibit the expected M_r of 212 by LC−MS (see Supporting Information). It can be assumed that **10** was *S*-(3-pyrrolyl) 3-pyrrolethiosulfinate formed by condensation of two molecules of 3-pyrrolesulfenic acid (Figure 5). Unlike the extremely reactive isomer **6**, thiosulfinate **10** appeared to be reasonably stable under experimental conditions, not transforming into any colored compounds.

In the next stage, we focused our attention on isolation and identification of the orange-red compound(s) formed upon crushing *A. giganteum* bulbs. The pigment was extracted from a bulb homogenate by diethyl ether. HPLC analysis of the extract revealed the abundant presence of a compound exhibiting a UV−vis absorption maximum at 519 nm (**11**). This compound was found to have identical UV−vis and ESI-MS spectra to those of the red product formed in model mixtures consisting of 2-PyrrCSO and alliinase. Thus, we attempted to obtain this compound by preparative HPLC. Despite using very mild conditions (e.g., freeze-drying), the collected material partially decomposed, rendering conventional NMR measurements impossible. Due to the profound instability of **11**, the structure of this compound was determined by means of LC−NMR and LC−HRMS. The ESI-HRMS data indicated the molecular formula of $C_8H_6N_2S_2$ ($[M - H]^-$, calcd for $C_8H_5N_2S_2$ 192.9900, found 192.9898). The ^{1}H LC−NMR spectrum consisted of only two doublets belonging to a pair of mutually coupled aromatic hydrogens (δ 6.36 and 6.48 ppm, J = 3.6 Hz, in D_2O/CD_3CN). These spectroscopic data are consistent with those reported by Jedelská et al. (δ 6.28 and 6.43 ppm, J = 3.7 Hz, in CD_3OD) for the red compound (λ_{max} at 518 nm) they isolated from *A. giganteum*.[4] They identified this compound as 3,3′-epidithio-2,2′-dipyrrole, assuming its precursor to be 3-PyrrCSO. Their proposal was based on comparison of experimental NMR data with those predicted by an NMR shift predictor for several possible isomeric structures. Based on our current findings that the precursor is in fact 2-PyrrCSO, it can be expected that **11** is rather 2,2′-epidithio-3,3′-dipyrrole (dipyrrolo[2,3-*d*:2′,3′-*e*]-1,2-dithiin). It can be proposed that **11** is formed from *S*-(2-pyrrolyl) 2-pyrrolethiosulfinate (**6**) via facile [3,3]-sigmatropic rearrangement in a similar fashion to the rearrangement of *S*-(1-propenyl) 1-propenethiosulfinate in cut onion[23,24] (Figure 6).

Two other abundant components present in the ether extract were identified as di(2-pyrrolyl) disulfide (**12**) and *S*-(2-pyrrolyl) 2-pyrrolethiosulfonate (**13**). The identity of **12** was deducted from ESI-HRMS data ($[M - H]^-$, calcd for $C_8H_7N_2S_2$ 195.0056, found 195.0049) and by comparison with an authentic sample obtained by synthesis. On the other hand, the structure of **13** was proposed only from the ESI-HRMS data ($[M - H]^-$, calcd for $C_8H_7N_2O_2S_2$ 226.9954, found 226.9957) and ESI-MS fragmentation patterns and should be considered as tentative. Disulfides and thiosulfonates are typically found in extracts obtained from various *Allium* species under relatively harsh conditions (e.g., by steam-distillation) and are thought to be formed by heat-induced disproportionation of the corresponding thiosulfinates. However, compounds **12** and **13** were detected in abundance not only in fresh bulb extracts prepared under mild

Table 2. *Allium giganteum* PI-DART and NI-DART Measurements

compd	species	calcd	found	diff (ppm)
	PI-DART			
6	$[C_8H_8N_2OS_2 + H]^+$	213.0151	213.0146	−2.4
7/8	$[C_5H_7NOS_2 + H]^+$	162.0042	162.0038	−2.6
11	$[C_8H_6N_2S_2 + H]^+$	195.0045	195.0041	−2.1
	NI-DART			
5	$[C_4H_4NOS]^-$	114.0019	114.0016	−2.6

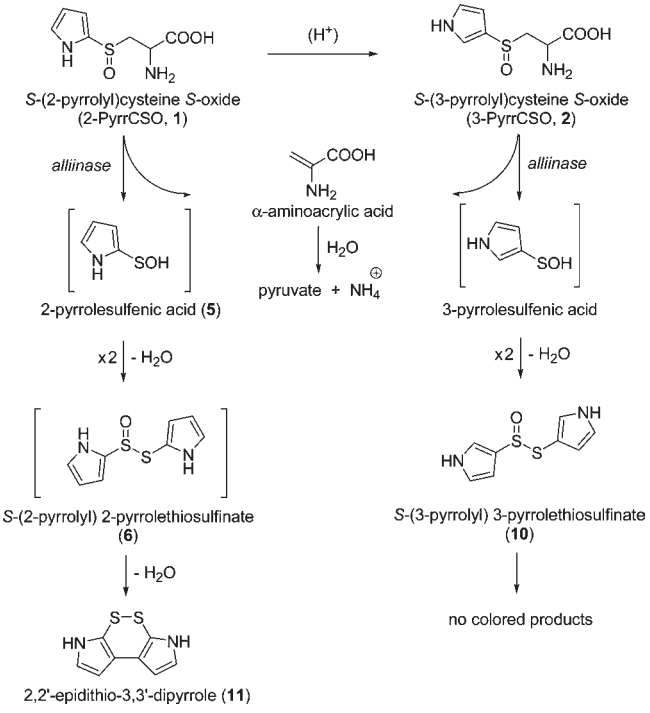

Figure 5. Alliinase-mediated decomposition of *S*-(2-pyrrolyl)- and *S*-(3-pyrrolyl)cysteine *S*-oxides.

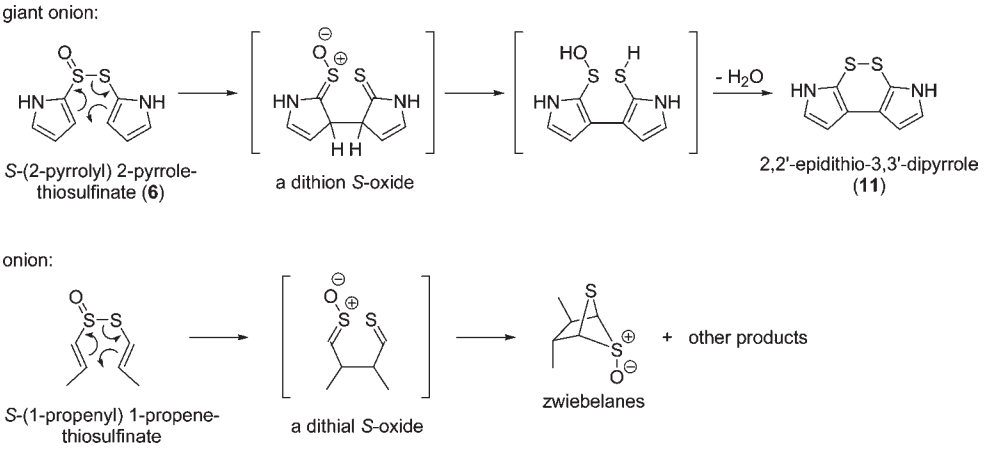

Figure 6. Proposed formation of 2,2′-epidithio-3,3′-dipyrrole in giant onion and the formation of zwiebelanes in onion.

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conditions but also in model mixtures consisting of 2-PyrrCSO and alliinase. These observations suggest that **12** and **13** are formed spontaneously in cut *A. giganteum* bulbs, although their formation pathways remain unclear.

It should also be noted that the deep orange ether extract from a bulb homogenate usually turned cloudy within several minutes on standing at room temperature and a precipitate formed at the bottom of the flask, indicating that some components present in the extract could readily undergo further transformations. Furthermore, both **1** and **3** appeared to be very sensitive to elevated temperature, readily decomposing during the isolation procedure to give rise to red colored degradation products. Even when the temperature during evaporation was strictly maintained below 30 °C, the color of both compounds became slightly pinkish. Both compounds also gradually decomposed upon storing at −28 °C and their color changed to orange-brown after approximately 3 months. On the other hand, 3-PyrrCSO appeared to be much more stable and did not show any signs of decomposition during storing at −28 °C for several weeks. Thus, it can be assumed that the pigment in *Melanocrommyum* species can be formed not only under the catalysis of alliinase but also by nonenzymatic degradation of **1** and **3**.

Although the ability to form the red pigment is quite widespread among subgenus *Melanocrommyum* species, it is not common to all of them. We observed that the pigment was formed upon wounding the bulbs of the following species: *A. giganteum*, *A. macleanii*, *A. sarawaschanicum*, *A. fetisowii*, *A. darwasicum*, *A. protensum*, and *A. newskianum*. On the other hand, the bulbs of *A. stipitatum*, *A. altissimum*, *A. cupuliferum*, or *A. rosenbachianum* did not produce any red pigment when cut. The species lacking the ability to form the pigment apparently do not synthesize the precursor, 2-PyrrCSO. This distinct biochemical feature of various *Melanocrommyum* species seems to be an important marker which may help in taxonomic classification of the subgenus.

The biochemical role of **1** and **3** in subgenus *Melanocrommyum* species is unclear. It is generally assumed that *S*-substituted cysteine derivatives in alliaceous species serve as storage compounds for sulfur or nitrogen. These amino acids, being precursors of an extraordinary variety of compounds, also play an important role in defense mechanisms of many plants. Unlike numerous methyl/allyl/1-propenyl/propyl analogues occurring in garlic and onion, the pyrrolyl compounds enzymatically formed from 2-PyrrCSO do not seem to be sufficiently volatile, pungent or lachrymatory to serve as attractants for pollinators or as repulsive compounds to deter predators (insect, ruminants). Thus, **1**, **3** and the compounds formed from them probably do not immediately discourage predators from attacking *A. giganteum* or other subgenus *Melanocrommyum* species. However, these compounds are likely to exhibit antimicrobial activity, thus they can effectively protect the wounded site against attacks of various pathogens. It was also observed that cells surrounding the transportation vessels produced higher amounts of the red pigment than other parts of the plant, indicating that 2-PyrrCSO and products of its transformations could protect the vessels transporting nutrients from possible damage.[4]

It can be concluded that the formation of the orange-red pigment in *A. giganteum* and some other subgenus *Melanocrommyum* species shares several common features with pinking of onion homogenates (Figure 2). The formation of both pigments is initiated by alliinase-catalyzed cleavage of *S*-substituted cysteine *S*-oxide precursors [*S*-(2-pyrrolyl)cysteine *S*-oxide and *S*-(*E*)-(1-propenyl)cysteine *S*-oxide (isoalliin), respectively] following tissue disruption. In both cases, alliinase-mediated cleavage of the respective precursor yields a thiosulfinate which can readily undergo [3,3]-sigmatropic rearrangement to form reactive dithiocarbonyl *S*-oxide intermediates (Figure 6). The structures and formation pathways of these two pyrrole-based pigments are however significantly different. Whereas the pigment in *Melanocrommyum* species seems to be formed solely by a spontaneous rearrangement of thiosulfinate **6**, pinking of onion homogenates is a more complex process, requiring the presence of a 1-propenyl-containing thiosulfinate, an amino compound and a (thio)carbonyl compound (Figure 2).

MATERIALS AND METHODS

General Methods. ^{1}H and ^{13}C NMR spectra of **1** and **2** were recorded on a Varian INOVA 500 MHz spectrometer, those of **3** were measured on a Varian Mercury 300 MHz spectrometer (Varian, Palo Alto, CA, USA). The chemical shifts were referenced externally to the signal of DSS. IR spectra were recorded on a Nicolet FTIR spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) and CD spectra on a Jasco J-715 circular dichroism spectrometer (Jasco, Tokyo, Japan). Specific rotation values were determined by means of an Autopol IV polarimeter (Rudolph Research Analytical, Hackettstown, NJ, USA). Melting points (uncorrected) were determined using a Stuart SMP 10 apparatus. HPLC separations were performed on a Dynamax SD-210 binary pump system (Varian, Palo Alto, CA, USA), employing a Varian PDA 335 detector and analytical C-18 or C-8 columns (Rainin Microsorb-MV 100 Å, 250 × 4.6 mm, 5 μm). Alternatively, a preparative C-8 column (Rainin Dynamax-100 Å, 250 × 21.4 mm, 8 μm) was used. A Varian ProStar 230 HPLC system (Varian, Palo Alto, CA, USA) was employed in the LC−NMR experiments. A PDA detector was used to detect chromatographic peaks which were then subjected to stop-flow ^{1}H NMR measurements conducted on a Varian INOVA 500 MHz spectrometer equipped with an H/C/N triple resonance microflow probe (60 μL active volume). ^{1}H LC−NMR data were collected in stop-flow mode employing WET multiple frequency solvent suppression. The data accumulation during 2 s acquisition time covering the spectral width of 10 kHz followed after 90° RF pulse (3.4 μs), the relaxation delay was set to 1 s. GC analyses were conducted on a Varian 3800 chromatograph (Varian, Palo Alto, CA, USA), equipped with a Varian 4000 MS detector. Samples (1 μL) were injected using a split ratio of 1:10 on an HP-5MS fused silica capillary column (30 m × 0.25 mm i.d.; film thickness 0.25 μm; Agilent Technologies, Santa Clara, CA, USA). The operating conditions employed were as follows: injector and detector temperatures of 180 and 250 °C, respectively; a helium carrier gas flow rate of 1.3 mL min^{-1}; a temperature linear gradient from 130 (3-min hold) to 220 at 2 °C min^{-1} was applied. ESI HRMS data were obtained by an LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) at resolution of R = 50 000 (fwhm), operating at 3.5 kV with ion source temperature of 200 °C. The DART-MS system consisted of a DART ion source (DART-SVP, IonSense, Saugus, MA, USA) coupled to an Exactive mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). The distance between the exit of the DART gun and the ceramic transfer tube was set to 10 mm, the gap between the ceramic tube and the inlet to the heated capillary of the Exactive mass spectrometer was 2 mm. Samples were introduced manually, employing Dip-It glass capillaries (IonSense, Saugus, MA, USA), the desorption time was approximately 5 s. The instrument was operated either in positive or negative ionization mode, with the following settings: helium flow, 2.5 L min^{-1}; gas temperature, 350 °C; discharge needle voltage, ± 5000 V; grid electrode, ± 350 V; MS detection, capillary voltage, ± 50 V; tube lens voltage, ± 120 V; capillary temperature, 250 °C. The mass resolving power of the instrument calculated for m/z 200 was R = 50 000 (fwhm).

Plant Material. The bulbs of various *Melanocrommyum* species were obtained from Dr. Leonid Bondarenko (Lithuanian Rare Bulb Garden, Vilnius, Lithuania) in October 2008. The bulbs of *A. giganteum* used for preparative work were purchased from Eurobulb (Zwanenburg, The Netherlands) in September 2008. Voucher specimens are still cultivated in the Alliaceae species collection at University of South Bohemia and can be accessed upon request.

Synthesis of Reference Compounds. *S*-Alk(en)yl-L-cysteine *S*-oxides were synthesized or isolated as described elsewhere.[18−21] 2-Pyrrolyl thiocyanate was obtained by thiocyanation of pyrrole according to Yadav et al.[25] Di(2-pyrrolyl) disulfide was prepared from 2-pyrrolyl thiocyanate by the procedure described in ref 26.

Isolation of Crude C−S Lyases (Alliinases). The procedure described by Shen and Parkin[27] was followed for the isolation of crude C−S lyases from the bulbs of *A. giganteum* and onion. The purity and specific activity of the obtained preparations were not examined in detail.

GC−MS and HPLC Analysis. *S*-Substituted cysteines present in the bulbs of various *Allium* subg. *Melanocrommyum* species were analyzed by the GC−MS method of Kubec et al.[21] The presence of the following derivatives was monitored: *S*-methyl-, *S*-ethyl-, *S*-propyl-, *S*-isopropyl-, *S*-allyl-, (*E*)-*S*-(1-propenyl)-, (*Z*)-*S*-(1-propenyl)-, *S*-butyl, *S*-isobutyl-, *S*-(*sec*-butyl)-, (*E*)-*S*-(1-butenyl)-, (*E*)-*S*-(2-butenyl)-, (*Z*)-*S*-(2-butenyl)-, *S*-(3-butenyl)-, *S*-pentyl-, *S*-(methylthiomethyl)-, *S*-phenyl-, and *S*-benzylcysteines. Quantitative determination was performed by HPLC after derivatization with dansyl chloride.[21]

Isolation of Compounds 1−3. Bulbs of *A. giganteum* (628 g) were cut in quarters and homogenized in 1 L of cold MeOH/H_2O/HCl (90/9/1, v/v/v), and the slurry was filtered through a layer of cotton wool. The extraction was repeated with another 1 L portion of cold MeOH/H_2O/HCl (90/9/1, v/v/v). The extracts were combined and concentrated to approximately 150 mL by vacuum evaporation (<35 °C). After centrifugation, the precipitate was disposed and the supernatant was adjusted to pH 2.5 by 5 M KOH and applied onto a cation-exchange column (22 × 3 cm; Amberlite IR-120, H^+ form, 16−45 mesh). After washing the column with H_2O (200 mL), the amino acid-containing fraction was eluted with 0.5 M NH_4OH. The Ehrlich's reagent-positive fractions were collected, their pH adjusted to 5.5−6.0 and freeze-dried. The residue obtained was redissolved in 25 mL of 50 mM KH_2PO_4 buffer (pH 5.5) and subjected to preparative C-8 HPLC, with 50 mM KH_2PO_4 (pH 5.5, solvent A) and acetonitrile (solvent B) as the mobile phase. The gradient was as follows: A/B 100/0 (0 min), 100/0 (in 4 min), 97/3 (in 6 min), 40/60 (in 8 min), 40/60 (in 12 min), and 100/0 (in 15 min), with a flow rate of 18 mL min^{-1}. The fractions eluting at 4.3, 7.2, and 8.8 min were collected, pooled, and freeze-dried. The residues obtained were extracted with 2 × 100 mL of MeOH, filtered and the combined extracts were carefully evaporated (<30 °C) to yield **2** (83 mg), **1** (552 mg) and **3** (109 mg), respectively. Partially contaminated **2** was further purified by passing the fraction through a column of Dowex 1 × 8 (25 × 2 cm, acetate form, 200−400 mesh) to obtain 27 mg of a colorless solid.

Attempted Isolation of Compound 11. Bulbs of *A. giganteum* (372 g) were cut in quarters and homogenized in 600 mL of H_2O. The homogenate was allowed to stand at room temperature for 30 min. Diethyl ether (1 L) was added to the already orange homogenate, and the resulting slurry was filtered through a layer of cotton wool. The extraction was repeated with another 1 L portion of diethyl ether, the extracts were combined and the layers were separated by centrifugation. The aqueous layer was re-extracted with 500 mL of ether, and the combined ether portions were dried over $MgSO_4$ and concentrated to dryness by vacuum evaporation (<30 °C). The dark orange solid obtained was redissolved in 30 mL of CH_3CN, filtered through a syringe-tip PTFE filter (0.45 μm), and passed through a short SPE C-8 column (100 mg, Supelco). Acetonitrile was removed by vacuum evaporation (<30 °C) to yield 455 mg of a deeply orange-red solid. The extract was subjected to preparative C-8 HPLC (1 mL injection loop), with H_2O (solvent A) and acetonitrile (solvent B) as the mobile phase. The gradient was as follows: A/B 60/40 (0 min), 56/44 (in 8 min), 5/95 (in 15 min), 5/95 (in 20 min), and 60/40 (in 25 min), with a flow rate of 18 mL min^{-1}. The fraction eluting at 18.1 min was collected, pooled and freeze-dried to yield 15 mg of a red solid. However, subsequent HPLC analysis of this fraction revealed that **11** partially decomposed giving rise to several products.

Model Experiments. Aliquots (1 mL) of stock solutions of **1** and **4** (25 mM in 50 mM KH_2PO_4 buffer, pH 6.5) were placed in 10 mL glass vials and mixed with 0.5 mL of an alliinase solution (10 mg/1 mL). The solutions were incubated with stirring at 23 °C for 30 min and extracted with 3 mL of diethyl ether and the organic portions stripped off using argon. The residues obtained were redissolved in acetonitrile (200 μL), filtered through a syringe-tip PTFE filter (0.45 μm) and analyzed by C-8 HPLC with H_2O (solvent A) and acetonitrile (solvent B) as the mobile phase. The gradient was as follows: A/B 95/5 (0 min), 5/95 (in 20 min), and 95/5 (in 25 min), with a flow rate of 0.9 mL min^{-1}. Similar experiments were performed with 3-PyrrCSO (**2**).

Analytical Data of the Identified Compounds. *(R_S,R_C)-S-(2-Pyrrolyl)cysteine S-oxide (2-PyrrCSO, **1**):* colorless solid; mp not determined (sample decomposed before melting); $[\alpha]_D^{22}$ +36.2° (H_2O); CD $\Delta\varepsilon_{max}$ (22 °C, H_2O) +3.24 (251 nm); UV (PDA, rel. int.) 250 nm (1.00), 219 nm (0.77); ^{1}H NMR (D_2O, 500 MHz) and ^{13}C NMR (D_2O, 125 MHz), see Table 1 and Supporting Information; IR (KBr) 3205, 1616, 1427, 1350, 1080, 995 cm^{-1}; ESI-MS, see Supporting Information; ESI-TOF HRMS calculated for $C_7H_{11}N_2O_3S$ 203.0485 $[M + H]^+$, found 203.0485; calculated for $C_7H_{10}N_2NaO_3S$ 225.0304 $[M + Na]^+$, found 225.0304; calculated for $C_7H_{10}KN_2O_3S$ 241.0044 $[M + K]^+$, found 241.0043.

*(R_S,R_C)-S-(3-Pyrrolyl)cysteine S-oxide (3-PyrrCSO, **2**):* colorless solid; mp not determined (sample decomposed before melting); $[\alpha]_D^{22}$ +45.7° (H_2O); CD $\Delta\varepsilon_{max}$ (22 °C, H_2O) +3.57 (231 nm); UV (PDA, rel. int.) 202 nm (1.00), 232 nm (0.63); ^{1}H NMR (D_2O, 500 MHz) and ^{13}C NMR (D_2O, 125 MHz), see Table 1 and Supporting Information; IR (KBr) 3108−3012, 1651, 1589, 1485, 1419, 1014 cm^{-1}; ESI-MS, see Supporting Information; ESI-TOF HRMS calculated for $C_7H_{11}N_2O_3S$ 203.0485 $[M + H]^+$, found 203.0483; calculated for $C_7H_9N_2O_3S$ 201.0339 $[M - H]^-$, found 201.0336.

*(R_S,R_C)-3-(2-Pyrrolylsulfinyl)lactic acid (**3**):* colorless solid; mp not determined (sample decomposed before melting); $[\alpha]_D^{22}$ +6.3° (H_2O); CD $\Delta\varepsilon_{max}$ (22 °C, H_2O) +2.89 (249 nm); UV (PDA, rel. int.) 250 nm (1.00), 216 nm (0.66); ^{1}H NMR (D_2O, 300 MHz) and ^{13}C NMR (D_2O, 75 MHz), see Table 1 and Supporting Information; IR (KBr) 3290, 1647, 1084, 987, 864 cm^{-1}; ESI-MS, see Supporting Information; ESI-TOF HRMS calculated for $C_7H_8NO_4S$ 202.0180 $[M - H]^-$, found 202.0182.

*Dipyrrolo[2,3-d:2′,3′-e]-1,2-dithiin (2,2′-epidithio-3,3′-dipyrrole) (**11**):* UV−vis (PDA, rel. int.) 519 nm (1.00), 295 nm (0.64), 355 nm (0.53); LC−^{1}H NMR (D_2O/CD_3CN, 500 MHz) δ 6.36 (d, J = 3.5 Hz, 2H, H-4/H-4′), 6.48 (d, J = 3.7 Hz, 2H, H-5/H-5′); ESI-MS, see Supporting Information; ESI-TOF HRMS calculated for $C_8H_5N_2S_2$ 192.9900 $[M - H]^-$, found 192.9898.

*Di(2-pyrrolyl) disulfide (**12**):* UV (PDA, rel. int.) 312 nm (1.00), 218 nm (0.96); ESI-MS, see Supporting Information; ESI-TOF HRMS calculated for $C_8H_7N_2S_2$ 195.0056 $[M - H]^-$, found 195.0049.

*S-(2-Pyrrolyl) 2-pyrrolethiosulfonate (**13**):* UV (PDA, rel. int.) 288 nm (1.00), 225 nm (0.97); ESI-MS, see Supporting Information; ESI-TOF HRMS calculated for $C_8H_7N_2O_2S_2$ 226.9954 $[M - H]^-$, found 226.9957.

■ ASSOCIATED CONTENT

Supporting Information. NMR, IR, CD, UV−vis, and ESI-MS spectra of compounds **1**−**3** and selected NMR, UV−vis, and ESI-MS spectra of compounds **10**−**13**. This material is available free of charge via the Internet at http://pubs.acs.org.

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ABBREVIATIONS USED

ADEQUATE, adequately sensitive double quantum transfer experiment; CD, circular dichroism; COSY, correlation spectroscopy; DART, direct analysis in real time; DEPT, distortionless enhancement by polarization transfer; DSS, 4,4-dimethyl-4-silapentane-1-sulfonic acid; ESI, electrospray ionization; fwhm, full width at half-maximum; GC−MS, gas chromatography−mass spectrometry; HETCOR, heteronuclear chemical shift correlation; HMBC, heteronuclear multiple bond correlation; HPLC, high-performance liquid chromatography; HRMS, high-resolution mass spectrometry; HSQC, heteronuclear single quantum correlation; IR, infrared; LC−MS, liquid chromatography−mass spectrometry; MCSO, *S*-methylcysteine *S*-oxide (methiin); NI, negative ionization; NMR, nuclear magnetic resonance; NOESY, nuclear Overhauser effect spectroscopy; PDA, photo diode array; PI, positive ionization; PTFE, polytetrafluorethene; 2-PyrrCSO, *S*-(2-pyrrolyl)cysteine *S*-oxide; 3-PyrrCSO, *S*-(3-pyrrolyl)cysteine *S*-oxide; RF, radio frequency; SPE, solid phase extraction; subg., subgenus; TOF, time-of-flight; UV, ultraviolet; WET, water suppression enhanced through T1 effects.

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