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Precursors and Formation of Pyrithione and Other Pyridyl-Containing Sulfur Compounds in Drumstick Onion, *Allium stipitatum*

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Supporting Information

ABSTRACT: Two novel, structurally unusual cysteine derivatives were isolated from the bulbs of *Allium stipitatum* (*Allium* subg. *Melanocrommyum*) and shown to be *S*-(2-pyridyl)cysteine *N*-oxide and *S*-(2-pyridyl)glutathione *N*-oxide. The former compound is the first example of a naturally occurring alliinase substrate that contains an *N*-oxide functionality instead of the *S*-oxide group. In addition, *S*-methylcysteine *S*-oxide (methiin) and *S*-(methylthiomethyl)cysteine 4-oxide (marasmin) were found in the bulbs. Presented data suggest that the previously reported identification of *S*-(2-pyridyl)cysteine *S*-oxide was most likely erroneous. The alliinase-mediated formation of pyridyl-containing compounds following disruption of *A. stipitatum* bulbs was studied by a combination of HPLC-MS, HPLC-PDA, DART-MS, and NMR techniques. It was found that no pyridyl-containing thiosulfinates are present in homogenized bulbs in detectable quantities. Instead, various pyridine *N*-oxide derivatives are formed, including *N*-hydroxypyridine-2(1*H*)-thione (pyrithione), 2-(methyldithio)pyridine *N*-oxide, 2-[(methylthio)methyldithio]pyridine *N*-oxide, di(2-pyridyl) disulfide *N*-oxide, and di(2-pyridyl) disulfide *N*,*N*′-dioxide. This represents the first report of pyrithione formation as a natural product.

KEYWORDS: *Allium stipitatum*, *S*-(2-pyridyl)cysteine *N*-oxide, marasmin, sulfenic acid, DART-MS, pyrithione

INTRODUCTION

The subgenus *Melanocrommyum* (Webb & Berthel.) Rouy belongs to the largest groups within the genus *Allium* (Alliaceae). It comprises about 160 mostly perennial species native to arid regions of the Mediterranean, the Near and Middle East, northwestern China, Pakistan, and Central Asia. Many *Melanocrommyum* species are popular ornamentals thanks to very attractive inflorescences (e.g., *Allium macleanii* J. G. Baker, *Allium giganteum* Regel, *Allium christophii* Trautv., and *Allium stipitatum* Regel), whereas some are important food crops cultivated in Central Asia (e.g., *A. stipitatum* Regel and *Allium suworovii* Regel).[1,2]

A. stipitatum produces sturdy stalks 80−100 cm tall, each bearing a showy, fireworks-like cluster of star-shaped flowers (see the Supporting Information), hence its common name drumstick onion (this common name is usually used for the whole group of *Melanocrommyum* species producing drumstick-like inflorescences). The flowers are typically purple, although white varieties are also known. This species is native to the dry steppes of eastern Afghanistan, Pakistan, Pamir, and the Tien-Shan Mountains. In these regions, it is frequently collected in the wild or cultivated under the local name "anzur" and used as a condiment (typically as pickled bulbs). It should be noted, however, that the same local name is also used for several other closely related *Melanocrommyum* species, for example, *Allium rosenbachianum* or *A. suworovii*.[2,3]

Despite the fact that many *Melanocrommyum* species are frequently consumed and widely used in folk medicine,[3] only little attention has been paid to the research of sulfur compounds present in these plants. *A. stipitatum* attracted our attention thanks to the recent study of O'Donnell et al.,[4] who isolated three sulfur-containing pyridine *N*-oxides from the bulbs of this plant (Figure 1). The identity of these structurally unusual compounds was rigorously confirmed by spectroscopic methods and by synthesis of authentic samples. On the other hand, Kusterer et al.[5] reported detection of several 2-pyridyl sulfur compounds in homogenized *A. stipitatum* bulbs, none of which reportedly contained the *N*-oxide moiety (Figure 1). However, the identification of compounds described in the latter study was mainly based on LC-MS/MS data without comparison with authentic standards.

This study was aimed at resolving the puzzling controversy between the aforementioned reports of O'Donnell et al.[4] and Kusterer et al.[5] In this paper, we describe the isolation and full spectral characterization of two structurally unique heterocyclic *S*-substituted cysteine derivatives present in intact bulbs of *A. stipitatum*. Furthermore, alliinase-mediated formation of pyridyl-containing compounds following tissue disruption was studied in detail by a combination of HPLC-MS, HPLC-PDA, DART-MS, and NMR techniques.

MATERIALS AND METHODS

General Methods. ^{1}H, ^{13}C, and ^{15}N NMR spectra were recorded on a Varian INOVA 500 MHz spectrometer (Varian, Palo Alto, CA). Chemical shifts of **1** and **2** were referenced externally to the signal

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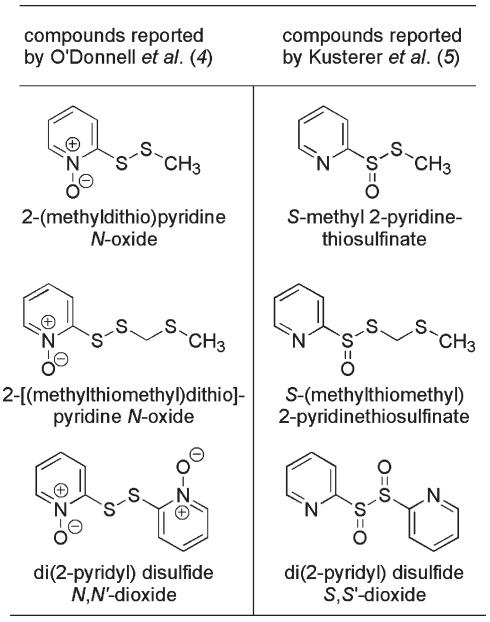

Figure 1. Structures of pyridyl-containing compounds previously reported to occur in *Allium stipitatum*.

of DSS. ^{1}H chemical shifts of compounds measured in $CDCl_3$ were referenced to the signal of HMDSS (δ 0.04) and ^{13}C shifts to the central line of the solvent signal (δ 76.99). ^{15}N NMR chemical shifts were referenced externally to the signal of nitromethane (δ 0.00) in the same deuterated solvent. ^{1}H chemical shifts of **15** measured by LC-NMR were referenced to the signal of CH_3CN (δ 2.00). Specific rotation values were recorded by means of an Autopol IV polarimeter (Rudolph Research Analytical, Hackettstown, NJ). Melting points (uncorrected) were determined using a Stuart SMP 10 apparatus. HPLC separations were performed on a Dynamax SD-210 binary pump system (Varian), employing a Varian PDA 335 detector and a C-8 column (Varian Microsorb-MV 100 Å, 250 × 4.6 mm, 5 μm). Alternatively, a preparative C-8 column (Varian Dynamax-100 Å, 250 × 21.4 mm, 8 μm) was used. LC-NMR, GC-MS, IR, ESI-TOF-HRMS, and DART-MS experiments were conducted using the instrumentation and settings as described in ref 5.

Plant Material. The bulbs of *A. stipitatum* 'Glory of Pamir' were obtained from Dr. Leonid Bondarenko (Lithuanian Rare Bulb Garden, Vilnius, Lithuania) in August 2009. The bulbs were originally collected in Hodja-obi-Garm (Hissar Mountains, Pamir, Tajikistan). For a picture of the plant see the Supporting Information. Voucher specimens are still cultivated in the Alliaceae species collection at University of South Bohemia and can be accessed upon request.

Chemicals. 2-Sulfanylpyridine *N*-oxide/*N*-hydroxypyridine-2(1*H*)-thione (**5**/**8**), pyridine-2-thiol, *S*-methyl methanethiosulfonate, L-cysteine, and glutathione were purchased from Acros Organics (Geel, Belgium). Chloromethyl methyl sulfide and 2-bromopyridine *N*-oxide hydrochloride were obtained from Sigma-Aldrich (St. Louis, MO), and acetonitrile was supplied by LabScan (Dublin, Ireland). Other chemicals were purchased from Spolana (Neratovice, Czech Republic).

Reference Compounds. (*R*)-*S*-(2-Pyridyl)cysteine *N*-oxide (**1**) and *S*-(2-pyridyl)glutathione *N*-oxide (**2**) were prepared by the reaction of 2-bromopyridine *N*-oxide with L-cysteine or glutathione as described in the Supporting Information. Various *S*-alk(en)yl-L-cysteine *S*-oxides were synthesized or isolated as described elsewhere.[6−11] 2-(Methyldithio)pyridine and 2-(methyldithio)pyridine *N*-oxide (**10**) were prepared as described in ref 4. Di(2-pyridyl) disulfide *N*-oxide (**15**) and di(2-pyridyl) disulfide *N*,*N*′-dioxide (**16**) were synthesized according to ref 12. However, di(2-pyridyl) disulfide *N*-oxide (**15**) appeared to be quite labile, readily disproportionating into **16** and di(2-pyridyl) disulfide (all attempts to purify it by preparative C-8 HPLC or silica gel chromatography failed). 2-[(Methylthiomethyl)dithio]pyridine *N*-oxide (**11**) was prepared by oxidation of equimolar amounts of (methylthio)methanethiol and 2-sulfanylpyridine *N*-oxide by hydrogen peroxide in acetone and subsequent purification by preparative HPLC chromatography. 2-[(Methylthiomethyl)dithio]pyridine was prepared analogously to the synthesis of **11**, starting from (methylthio)methanethiol and pyridine-2-thiol.

Isolation of Crude C−S Lyases (Alliinases). The procedure described in ref 13 was followed for the isolation of crude C−S lyases from the bulbs of *A. stipitatum* 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 *A. stipitatum* were analyzed by the GC-MS and HPLC methods of Kubec and Dadáková.[9] The presence of the 18 various cysteine derivatives listed in ref 6 was monitored.

Isolation of Compounds 1 and 2. Bulbs of *A. stipitatum* (337 g) were cut in quarters and finely homogenized in 1 L of MeOH/H_2O/HCl (90:9:1, v/v/v), and the slurry was allowed to gently boil under reflux for 5 min and filtered through a layer of cotton wool. The extraction was repeated with another 1 L portion of 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 discarded, 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 the column had been washed with H_2O (100 mL), the amino acid-containing fraction was eluted with 0.5 M NH_4OH. The ninhydrin-positive fractions were collected, their pH was adjusted to 5.5−6.0, and then they were freeze-dried. The residue obtained was redissolved in 20 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 97:3 (0 min), 97:3 (in 5 min), 40:60 (in 10 min), 40:60 (in 15 min), and 97:3 (in 22 min), with a flow rate of 18 mL min^{-1}. The fractions eluting at 7.2 and 11.3 min were collected, pooled, and freeze-dried. The residues obtained were extracted with 2 × 150 mL of MeOH and filtered, and the combined extracts were carefully evaporated (<30 °C) to yield **1** (569 mg) and **2** (50 mg), respectively.

Extraction of Enzymatically Formed Compounds. Bulbs of *A. stipitatum* (94.4 g) were cut in quarters and finely homogenized in 150 mL of H_2O using a blender. The homogenate was allowed to stand at room temperature for 30 min. Diethyl ether (200 mL) was added to the homogenate, and the resulting slurry was filtered through a layer of cotton wool. The layers were separated by centrifugation, and the aqueous layers was extracted with another 100 mL portion of diethyl ether. The combined ether portions were dried over $MgSO_4$ and concentrated to dryness by vacuum evaporation (<23 °C). The residue obtained was redissolved in 1 mL of CH_3CN, 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 90:10 (0 min), 85:15 (in 10 min), 10:90 (in 40 min), 10:90 (in 50 min), and 90:10 (in 55 min), with a flow rate of 0.9 mL min^{-1}.

Model Experiments. Aliquots (1 mL) of stock solutions of **1**, **3** 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 a crude alliinase solution (10 mg/1 mL). The model mixtures were incubated with stirring at 23 °C for 30 min (in some experiments only 2 min) and extracted with 3 mL of CH_2Cl_2, and the organic portion was stripped off using argon. The residues obtained were redissolved in acetonitrile (300 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, emloying the same solvent gradient as described above. For DART-MS experiments, analogous model mixtures were prepared, incubated for

1 min, and analyzed without filtration or extraction. When alliinase-mediated cleavage of **1** was studied by ^{1}H NMR, 10 mg of **1** in 0.3 mL of D_2O was mixed with 0.4 mL of a crude alliinase solution (10 mg/1 mL) directly in an NMR tube. After shimming, the ^{1}H NMR spectrum was recorded.

Analytical Data of the Identified Compounds. *(R)-S-(2-Pyridyl)cysteine N-oxide (2-PyCNO, **1**):* colorless solid; mp 179−181 °C; $[\alpha]_D^{22}$ +29.4° (H_2O); UV (PDA, rel int) 237 nm (1.00), 262 nm (sh, 0.33), 304 nm (0.11); ^{1}H NMR (D_2O, 500 MHz) δ 3.38 (dd, 1H, J = 14.7 and 8.5 Hz, H-3a), 3.62 (dd, 1H, J = 14.7 and 3.9 Hz, H-3b), 3.95 (dd, 1H, J = 8.5 and 3.9 Hz, H-2), 7.27 (ddd, 1H, J = 7.5, 6.5, and 1.5 Hz, H-5′), 7.52 (ddd, 1H, J = 8.0, 1.5, and 1.1 Hz, H-3′), 7.58 (ddd, 1H, J = 8.0, 7.5, and 0.6 Hz, H-4′), 8.22 (ddd, 1H, J = 6.5, 1.1, and 0.6 Hz, H-6′); ^{13}C NMR (D_2O, 125 MHz) δ 31.8 (C-3), 53.3 (C-2), 122.6 (C-5′), 123.6 (C-3′), 131.7 (C-4′), 139.5 (C-6′), 150.6 (C-2′), 173.1 (C-1); ^{15}N NMR (D_2O, 50.7 MHz) δ −342.8 (NH_2), −115.5 (N═O); IR (KBr) 3090−2540, 1628, 1574, 1361, 1219, 833, 764 cm^{-1}. ESI-TOF HRMS: calcd for $C_8H_{11}N_2O_3S$, 215.0485 $[M + H]^+$; found, 215.0485 (for NMR, IR, ESI-MS, and UV spectra see the Supporting Information).

*(S_{C2},R_{C7})-S-(2-Pyridyl)glutathione N-oxide (**2**):* colorless hygroscopic solid; $[\alpha]_D^{22}$ −6.5° (H_2O); UV (PDA, rel int) 238 nm (1.00), 261 nm (sh, 0.39), 307 nm (0.15); ^{1}H NMR (D_2O, 500 MHz) δ 1.89 (dd, 2H, J = 14.5 and 7.5 Hz, H-3), 2.33 (dt, 2H, J = 7.5 and 3.0 Hz, H-4), 3.30 (dd, 1H, J = 14.3 and 8.8 Hz, H-12a), 3.45 (t, 1H, J = 5.9 Hz, H-2), 3.54 (dd, 1H, J = 14.3 and 5.1 Hz, H-12b), 3.62 (q, 2H, J = 17.2 Hz, H-10), 4.68 (dd, 1H, J = 9.0 and 4.9 Hz, H-7), 7.25 (ddd, 1H, J = 7.6, 6.5, and 1.6 Hz, H-5′), 7.52 (ddd, 1H, J = 8.4, 1.6, and 0.4 Hz, H-3′), 7.56 (ddd, 1H, J = 8.4, 7.6, and 1.3 Hz, H-4′), 8.20 (ddd, 1H, J = 6.5, 1.3, and 0.4 Hz, H-6′); ^{13}C NMR (D_2O, 125 MHz) δ 27.7 (C-3), 31.6 (C-12), 31.8 (C-4), 43.6 (C-10), 52.0 (C-7), 54.7 (C-2), 122.3 (C-5′), 123.4 (C-3′), 131.5 (C-4′), 139.4 (C-6′), 151.3 (C-2′), 171.2 (C-8), 175.5 (C-5), 176.4 (C-11), 176.7 (C-1); IR (KBr) 3530−2650, 1658, 1608, 1419, 1222, 837 cm^{-1}. ESI-TOF HRMS: calcd for $C_{15}H_{21}N_4O_7S$, 401.1125 $[M + H]^+$; found, 401.1127; calcd for $C_{15}H_{19}N_4O_7S$, 399.0980 $[M - H]^-$; found, 399.0981 (for NMR, IR, ESI-MS, and UV spectra see the Supporting Information).

*2-(Methyldithio)pyridine N-oxide (**10**):* colorless solid; mp 107−110 °C; UV (PDA, rel int) 237 nm (1.00), 264 nm (sh, 0.41), 303 nm (sh, 0.12); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 2.45 (s, 3H, CH_3), 7.15 (ddd, 1H, J = 7.6, 6.4, and 1.8 Hz, H-5′), 7.38 (ddd, 1H, J = 8.3, 7.6, and 1.2 Hz, H-4′), 7.87 (ddd, 1H, J = 8.3, 1.8, and 0.5 Hz, H-3′), 8.25 (ddd, 1H, J = 6.4, 1.2, and 0.5 Hz, H-6′); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 21.7 (CH_3), 121.3 (C-3′), 121.6 (C-5′), 126.2 (C-4′), 138.7 (C-6′), 151.7 (C-2′); ^{15}N NMR ($CDCl_3$, 50.7 MHz) δ −97.6; IR (KBr) 1466, 1423, 1246, 1142, 833, 752 cm^{-1}. ESI-TOF HRMS: calcd for $C_6H_8NOS_2$, 174.0042 $[M + H]^+$; found, 174.0041 (for NMR, IR, ESI-MS, and UV spectra see the Supporting Information).

*2-[(Methylthio)methyldithio]pyridine N-oxide (**11**):* yellow oil; UV (PDA, rel int) 239 nm (1.00), 265 nm (sh, 0.51), 304 nm (sh, 0.14); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 2.31 (s, 3H, CH_3), 3.88 (s, 2H, CH_2), 7.15 (ddd, 1H, J = 7.6, 6.4, and 1.8 Hz, H-5′), 7.36 (ddd, 1H, J = 8.6, 7.6, and 1.2 Hz, H-4′), 7.90 (dd, 1H, J = 8.3 and 1.8 Hz, H-3′), 8.24 (ddd, 1H, J = 6.4, 1.2, and 0.5 Hz, H-6′); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 15.8 (CH_3), 43.7 (CH_2), 121.6 (C-3′), 121.8 (C-5′), 126.0 (C-4′), 138.6 (C-6′), 151.6 (C-2′); ^{15}N NMR ($CDCl_3$, 50.7 MHz) δ −97.5; IR (KBr) 1462, 1423, 1261, 836, 760 cm^{-1}. ESI-TOF HRMS: calcd for $C_7H_{10}NOS_3$, 219.9919 $[M + H]^+$; found, 219.9920; calcd for $C_7H_9NNaOS_3$, 241.9739 $[M + Na]^+$; found, 241.9738 (for NMR, IR, ESI-MS, and UV spectra see the Supporting Information).

*Di(2-pyridyl) disulfide N-oxide (**15**):* UV (PDA, rel int) 236 nm (1.00), 266 nm (0.80), 308 nm (sh, 0.18); LC-^{1}H NMR (D_2O/CH_3CN, 500 MHz) δ 7.31 (t, 1H, J = 5.8 Hz, H-5), 7.39 (t, 1H, J = 6.5 Hz, H-5′), 7.56−7.62 (m, 2H, H-4/H-4′), 7.76−7.82 (m, 2H, H-3/H-3′), 8.32 (d, 1H, J = 6.2 Hz, H-6), 8.40 (s, 1H, H-6′). ESI-TOF HRMS: calcd for $C_{10}H_9N_2OS_2$, 237.0151 $[M + H]^+$; found, 237.0150; calcd for $C_{10}H_8N_2NaOS_2$, 258.9970 $[M + Na]^+$; found, 258.9971 (for NMR, ESI-MS, and UV spectra see the Supporting Information).

*Di(2-pyridyl) disulfide N,N′-dioxide (**16**):* colorless solid; mp 215−218 °C; UV (PDA, rel int) 236 nm (1.00), 262 nm (sh, 0.47), 302 nm (sh, 0.15); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.19 (ddd, 2H, J = 7.8, 6.4, and 1.8 Hz, H-5/H-5′), 7.28 (ddd, 2H, J = 8.2, 7.8, and 1.1 Hz, H-4/H-4′), 7.60 (ddd, 2H, J = 8.2, 1.8, and 0.5 Hz, H-3/H-3′), 8.30 (ddd, 2H, J = 6.5, 1.1, and 0.5 Hz, H-6/H-6′); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 122.9 (C-3/C-3′), 123.5 (C-5/C-5′), 127.5 (C-4/C-4′), 139.6 (C-6/C-6′), 150.2 (C-2/C-2′); ^{15}N NMR ($CDCl_3$, 50.7 MHz) δ −96.0; IR (KBr) 1462, 1419, 1250, 1227, 837, 764 cm^{-1}. ESI-TOF HRMS: calcd for $C_{10}H_9N_2O_2S_2$, 253.0100 $[M + H]^+$; found, 253.0100; calcd for $C_{10}H_8N_2NaO_2S_2$, 274.9919 $[M + Na]^+$; found, 274.9920 (for NMR, IR, ESI-MS, and UV spectra see the Supporting Information).

RESULTS AND DISCUSSION

An amino acid/oligopeptide-containing fraction from the bulbs of *A. stipitatum* was obtained by extraction with acidified aqueous methanol 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 230−260 nm. Two of these compounds were subsequently isolated by preparative C-8 HPLC and fully characterized by spectroscopic methods.

^{13}C NMR data of the major isolated compound (**1**) indicated the presence of five magnetically different aromatic carbons, together with two sp^3-hybridized carbon atoms and one carboxylic group. Further NMR experiments (including COSY, HETCOR, and HMBC) revealed the presence of two isolated structural subunits: (i) a *C*-monosubstituted pyridyl moiety and (ii) a $-CH_2CH(X)COOH$ chain. Unambiguous evidence that the pyridyl moiety was substituted at position 2 was obtained by a detailed analysis of the ^{1}H spin system and from ^{1}H NMR spectra measured with selective proton decoupling. These spectra showed that the four aromatic hydrogens formed a coupling pathway chain with each of the two internal hydrogens being vicinally coupled ($J \sim 7$ Hz) to two other hydrogens. Those at δ 8.22 and 7.52 terminated the chain as they were coupled to only one neighboring hydrogen (see the Supporting Information). The ESI-TOF HRMS exhibited $[M + H]^+$ of 215.0485 (calcd for $C_8H_{11}N_2O_3S$, 215.0485), indicating that the compound was an oxide of *S*-(2-pyridyl)cysteine. The most difficult task in the structure elucidation of **1** was to determine the site of oxidation. Two possible isomeric structures were taken into consideration, namely, *S*-(2-pyridyl)cysteine *S*-oxide and *S*-(2-pyridyl)cysteine *N*-oxide (Figure 2). The abundant and widespread occurrence of various *S*-substituted cysteine *S*-oxides in alliaceous plants initially favored the *S*-oxide structure. However, the ^{13}C and ^{15}N NMR shifts together with the IR spectrum of **1** suggested that the compound was rather the *N*-oxide. The most indicative of correct assignment was the ^{13}C NMR shift of the carbon C-3. It has been observed that ^{13}C NMR shifts of C-3 carbons in various *S*-substituted cysteine *S*-oxides vary only slightly between δ 50 and 55, whereas those in *S*-substituted cysteines appear in the region of δ 31−35 (in D_2O). It is noteworthy that the *S*-bound substituent has only a marginal effect on the shift (see the Supporting Information). The signal of C-3 in **1** was found at δ 31.8, indicating that the sulfur was not oxidized. This conclusion was further supported by the fact that **1** was not cleaved by onion alliinase, which is known to act only upon *S*-oxide derivatives of L-cysteine[14] [e.g., *S*-(2-pyrrolyl)cysteine *S*-oxide was readily cleaved by the same crude onion alliinase preparation[6]].

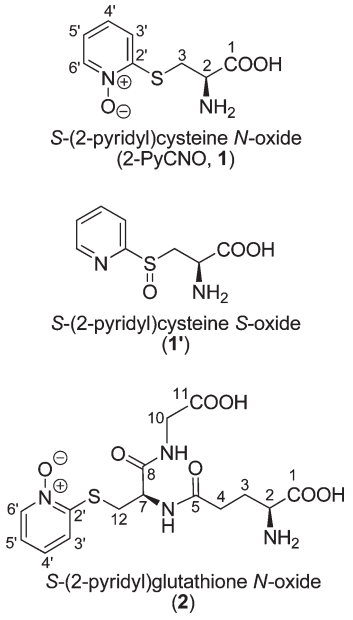

Figure 2. Structures of pyridyl-containing amino compounds isolated from *Allium stipitatum* in this study (**1** and **2**) and the compound (**1′**) reportedly isolated by Kusterer et al.[5]

Furthermore, the IR spectrum contained no significant absorption band in the region of 980−1040 cm^{-1} (−S═O). Foolproof evidence about the identity of **1** was obtained by its comparison with an authentic sample of (*R*)-*S*-(2-pyridyl)-cysteine *N*-oxide prepared by synthesis. The compound isolated from the bulbs was found to be identical in all respects (NMR, MS, UV, optical rotation) with the standard. Therefore, the structure of **1** could be unambiguously assigned as (*R*)-*S*-(2-pyridyl)cysteine *N*-oxide (2-PyCNO) (Figure 2).

Kusterer et al.[5] recently reported the isolation of an OPA-derivatized amino acid from *A. stipitatum* that they claimed to be *S*-(2-pyridyl)cysteine *S*-oxide (**1′**). However, we did not detect any other compound with M_r 214 except for **1** in the amino acid fraction by HPLC-MS. Careful evaluation of the NMR data given by Kusterer et al. for the compound they isolated revealed a very close similarity of its ^1H and ^{13}C NMR shifts to those of **1**. In particular, the ^{13}C NMR shift of carbon C-3 in the OPA derivative was δ 35.5 (in CD_3CN), clearly indicating that the compound was not an *S*-oxide. Thus, we believe that the compound isolated by Kusterer et al. was in fact the OPA derivative of *S*-(2-pyridyl)cysteine *N*-oxide (**1**). A great deal of attention was also paid to ruling out the possibility that **1** was formed from the putative **1′** during isolation. Therefore, a bulb of the plant was homogenized in cold methanol, and the extract obtained was directly analyzed by HPLC (without any exposure to elevated temperature and pH extremes). Indeed, **1** was the only component with M_r 214 present in the extract, clearly confirming that this amino acid is not an artifact formed from **1′** during isolation.

Herein, we report the isolation and full spectral characterization of pure **1** for the first time. It is noteworthy that 2-PyCNO (**1**) is only the third naturally occurring example of an aromatic *S*-substituted cysteine derivative. The other two compounds are *S*-benzylcysteine *S*-oxide (petiveriin) from the Amazonian plant *Petiveria alliacea* L. (Phytolaccaceae) and *S*-(2-pyrrolyl)cysteine *S*-oxide from giant onion (*A. giganteum* Regel) and some other subgenus *Melanocrommyum* species.[6,7] However, 2-PyCNO (**1**) is structurally markedly distinct from all derivatives previously isolated from alliaceous plants in that it contains the *N*-oxide moiety instead of the *S*-oxide group.

ESI-TOF HRMS data of the second compound (**2**) isolated from the extract corresponded to the molecular formula of $C_{15}H_{20}N_4O_7S$. The ^{13}C NMR spectrum showed the presence of 15 different carbon atoms, including 5 aromatic and 4 C═O carbons. Additional NMR experiments (COSY, HETCOR, HMBC, and HSQC) revealed the presence of several isolated structural subunits, namely, an *N*-oxy-2-pyridyl moiety and $-C(O)CH_2CH_2CH(X)COOH$ $-CH_2CH(X)CO-$ $-CH_2(X)COOH$ chains. These spectral data indicated the compound to be an *S*-substituted glutathione derivative, *S*-(2-pyridyl)glutathione *N*-oxide (Figure 2). Indeed, this tripeptide was subsequently prepared by synthesis and found to have spectral properties (NMR, MS, and UV) identical to those of **2**. Analogous *S*-substituted glutathione derivatives (bearing 2-carboxypropyl or methyl moieties) occur in onion, garlic, and other alliaceous species and are thought to be intermediates in the biogenesis of *S*-substituted cysteine *S*-oxides.[15] Thus, it seems to be reasonable to assume that **2** is a biochemical precursor of **1**.

GC-MS and HPLC analysis of the amino acid fraction isolated from *A. stipitatum* bulbs also revealed the presence of two other *S*-substituted cysteine derivatives, namely, (S_S,R_C)-*S*-methylcysteine *S*-oxide (methiin, MCSO, **3**) and (R_S,R_C)-*S*-(methylthiomethyl)cysteine 4-oxide (marasmin, MTMCSO, **4**). The identity and absolute configurations of **3** and **4** were confirmed by comparison with authentic standards. The relative ratio of **1**/**3**/**4** was found to be 51:17:32 in the bulbs of *A. stipitatum* we analyzed. Whereas methiin occurs abundantly in all alliaceous species,[6,8,9,11,16] the presence of marasmin has not thus far been reported in any genus *Allium* plant. This amino acid is the key odor precursor of society garlic (*Tulbaghia violacea* Harv.), an alliaceous plant belonging to the taxonomically closely related genus *Tulbaghia*, which comprises species native to South Africa.[10]

The alliinase-mediated conversion of the three cysteine derivatives concomitantly present in the intact bulbs (**1**, **3**, and **4**) was studied by a combination of HPLC-MS, HPLC-PDA, DART-MS, and ^1H NMR techniques. A diethyl ether extract of homogenized bulbs of *A. stipitatum* was prepared and immediately analyzed by HPLC-PDA. The chromatogram obtained was surprisingly very simple, revealing the presence of only a few compounds, with two of them accounting for 74% of the total peak area (at 210 nm; see the Supporting Information). HPLC-MS analysis of the extract indicated that the two most abundant components exhibited M_r of 173 and 219. These compounds were subsequently shown to be identical in all respects (chromatographic behavior, UV and MS spectra) with authentic samples of 2-(methyldithio)pyridine *N*-oxide (**10**) and 2-[(methylthiomethyl)dithio]pyridine *N*-oxide (**11**), respectively. In addition, *N*-hydroxypyridine-2(1*H*)-thione (**8**), di(2-pyridyl) disulfide *N*-oxide (**15**), and di(2-pyridyl) disulfide *N*,*N*′-dioxide (**16**) were identified as minor components in the extract by HPLC-MS and by comparison with standards. It should be mentioned that compounds **8**, **10**, **11**, **15**, and **16** appeared to be quite photolabile, which rendered the use of preparative HPLC for their isolation impossible (unless the effluent was diverted before the PDA detector cell). Also during freeze-drying or purification by silica gel column chromatography, most samples decomposed, giving rise to bluish degradation products.

It is noteworthy that no other compounds having a molecular weight of M_r 173, 219, 236, or 252 (corresponding to the presumable

Table 1. *Allium stipitatum* NI-DART and PI-DART Measurements

ionization	compound	species	calcd	found	rel abundance (%)
NI-DART	**6**	$[CH_3OS]^-$	62.9910	62.9898	0.49
	7	$[C_2H_5OS_2]^-$	108.9787	108.9776	0.56
	5/8	$[C_5H_4NOS]^-$	126.0019	126.0008	100
PI-DART	**9**	$[C_2H_6OS_2 + H]^+$	110.9933	110.9934	0.24
	13/14	$[C_3H_8OS_3 + H]^+$	156.9810	156.9810	nd[a]
	10	$[C_6H_7ONS_2 + H]^+$	174.0042	174.0042	100
	12	$[C_4H_{10}OS_4 + H]^+$	202.9687	202.9688	nd
	11	$[C_7H_9NOS_3 + H]^+$	219.9919	219.9920	7.31
	15	$[C_{10}H_8N_2OS_2 + H]^+$	237.0151	237.0150	0.38

[a] Not detected after puncturing the bulb (abundant in a model mixture consisting of **3**, **4**, and crude alliinase).

thiosulfinates and *vic*-disulfoxide reported by Kusterer et al.) were detected in the extract by HPLC-MS, despite our specifically searching for them. Even when various model mixtures consisting of **1**, **3**, and **4** were injected shortly (within 2 min) after mixing with crude alliinase from *A. stipitatum*, no such compounds were detected.

To get more detailed insight into the formation pathways of **8**−**16**, direct analysis in real time mass spectrometry (DART-MS) was also employed. This exceptionally mild desorption ionization technique allows one to observe the formation of compounds of only a fleeting existence without the necessity for any sample pretreatment, simply by momentarily holding the tested material in the DART gas stream.[6,11,17] A bulb of *A. stipitatum* was punctured by a sampling capillary, which was immediately (within 2−3 s after tissue disruption) inserted in the DART source ionization region. As shown in Table 1, the predominant signal detected by negative ionization (NI) DART-HRMS corresponded to a compound(s) with the molecular formula of C_5H_5NOS. It can be assumed that this compound was 2-sulfanylpyridine *N*-oxide (**5**) arising by alliinase-mediated cleavage of 2-PyCNO (**1**). Furthermore, signals corresponding to the two sulfenic acids **6** and **7** (formed from **3** and **4**, respectively) were also detected by NI-DART-HRMS. Positive ionization (PI) DART-HRMS experiments confirmed the abundant presence of **10** and **11**, along with minor amounts of **9** and **15** (see the Supporting Information). Similar results were also obtained by analyzing various model mixtures consisting of **1**, **3**, **4**, and crude alliinase from *A. stipitatum*. The alliinase-mediated cleavage of 2-PyCNO (**1**) was also studied by NMR. 2-PyCNO was mixed with crude alliinase directly in an NMR tube, and the ^{1}H NMR spectrum was recorded. 2-PyCNO was completely consumed within 3 min, and the only signals detected in the region of δ 6−9 were those belonging to *N*-hydroxypyridine-2(1*H*)-thione (**8**) (see the Supporting Information).

On the basis of the data obtained, it could be proposed that compounds **5**−**16** are formed as outlined in Figures 3 and 4. Upon tissue disruption, 2-PyCNO (**1**) is cleaved by alliinase to yield 2-sulfanylpyridine *N*-oxide (**5**), which in turn condenses with sulfenic acids **6** and **7**, giving rise to **10** and **11**, respectively. Indeed, when **5** was added into model mixtures consisting of crude alliinase and either **3** or **4**, the formation of **10** and **11**, respectively, was observed. Compound **5** can also spontaneously rearrange into tautomeric and more stable *N*-hydroxypyridine-2(1*H*)-thione (**8**). It has been reported that, in aqueous media, the thione form (**8**) predominates over the *N*-oxide (**5**), with the molar ratio of **8**/**5** being approximately 54.[18,19] The other pyridine *N*-oxides detected, di(2-pyridyl) disulfide *N*-oxide (**15**) and di(2-pyridyl) disulfide *N*,*N*′-dioxide (**16**), are probably formed by secondary transformations of **5** and **8**, as documented by numerous studies (Figure 4).[12,19−21] The assumption that **15** and **16** are not primary products of the alliinase-mediated cleavage of 2-PyCNO (**1**) is also supported by the facts that only low amounts of these two compounds were found in the ether extract by HPLC and the formation of **16** was not observed by DART-MS immediately after tissue disruption. To our surprise, no evidence was obtained about the expected presence of the three marasmin-derived thiosulfinates **12**−**14**, presumably formed by condensation of **6** and **7** (Figure 3). The presence of these thiosulfinates was not detected by HPLC-MS in the ether extract or by DART-MS upon puncture of the bulb. These compounds were found only in a model mixture consisting of **3**, **4**, and crude alliinase in the absence of **1** (Table 1). We therefore assume that, in the bulbs we analyzed, sulfenic acid **7** had been completely trapped by **5** before it could form the thiosulfinates.

Our results clearly show that no pyridyl-containing thiosulfinates are formed in detectable quantities in crushed *A. stipitatum* tissue. These findings are in major conflict with the study of Kusterer et al.,[5] who reported detection of several 2-pyridyl thiosulfinates and one *S*,*S*′-dioxide derivative (Figure 1). However, they deduced structures of these compounds mainly from HPLC-MS/MS data (without comparison with authentic samples), assuming the precursor to be *S*-(2-pyridyl)cysteine *S*-oxide (**1**′). The only compound they characterized more rigorously (by NMR and IR) was claimed to be di(2-pyridyl) disulfide *S*,*S*′-dioxide. However, the presence of this compound would be highly surprising, as *vic*-disulfoxides are known to be extremely reactive, nonisolatable intermediates of only a fleeting existence at room temperature.[22,23] The ^{1}H NMR data given by Kusterer et al.[5] for this compound were carefully evaluated and found to be nearly identical to those of di(2-pyridyl) disulfide *N*,*N*′-dioxide (**16**). Further evidence that the compounds described in ref 5 were in fact the corresponding *N*-oxides was obtained from their UV spectra. We observed that 2-pyridyl-containing disulfides exhibit considerably different UV spectra from those of their *N*-oxide counterparts. UV spectra of the former compounds contain an intense absorption band around 282 nm, whereas pyridine *N*-oxides do not show significant absorption in this region (see the Supporting Information). On the other hand, the latter compounds typically exhibit a maximum at 236−239 nm, with two considerably less intense shoulders around 262 and 304 nm (in H_2O/CH_3CN). All compounds detected by Kusterer et al. exhibited UV absorption at 238, 260−264, and 310 nm (in CH_3OH), clearly suggesting they were pyridine *N*-oxides rather

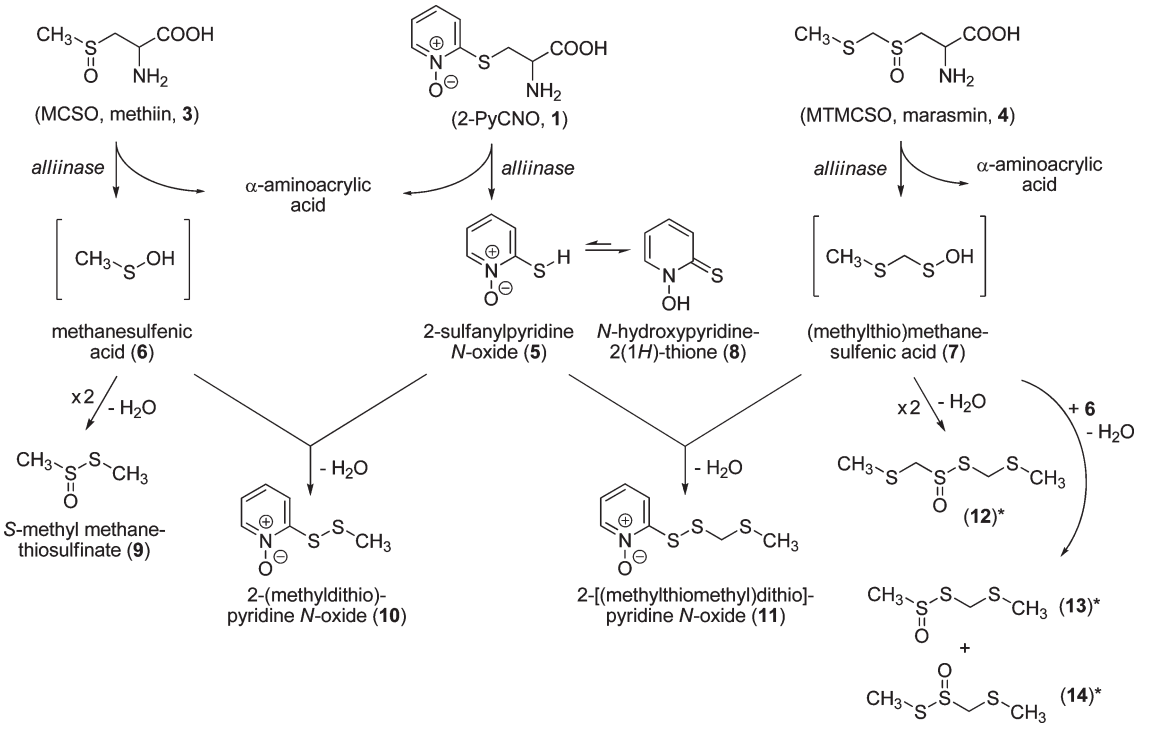

Figure 3. Alliinase-mediated formation of volatile compounds in *Allium stipitatum* (the compounds marked by ∗ were detected only in some model mixtures).

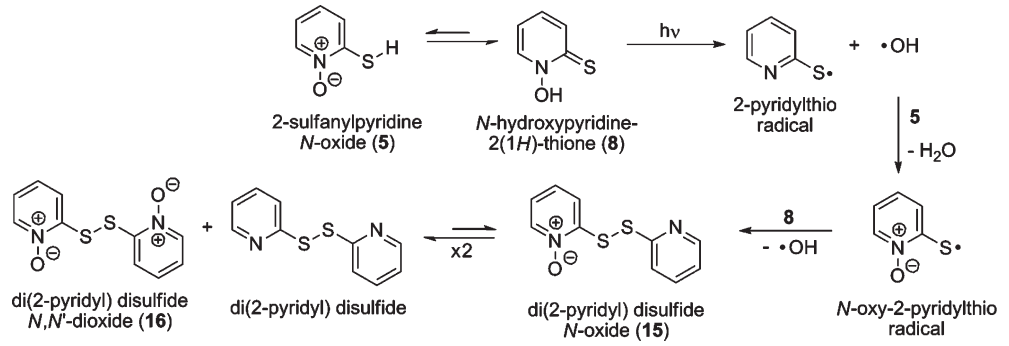

Figure 4. Proposed formation of pyridyl-containing compounds from pyrithione (adapted with permission from ref 20).

than thiosulfinates. Finally, if thiosulfinates had been formed, both regiomers differing in the position of the oxygen atom [i.e., RS(O)SR′ and RSS(O)R′] should have been observed. However, no such pairs of compounds were reported in ref 5.

Compounds **10**, **11**, and **16** were already isolated from *A. stipitatum* by O'Donnell et al.,[4] and **16** was also found in a basidiomycetous mushroom of the genus *Cortinarius* native to New Zealand.[24] These three compounds were shown to possess potent inhibitory activity against several multidrug-resistant strains of microorganisms including *Staphylococcus aureus*.[4] Of particular interest is the formation of *N*-hydroxypyridine-2(1*H*)-thione (pyrithione, **8**) upon crushing of the bulbs of *A. stipitatum*. To the best of our knowledge, this is the first report of pyrithione formation from a naturally occurring precursor (i.e., 2-PyCNO, **1**). This compound, a distant analogue of aspergillic acid, is known to exhibit significant antimicrobial properties[25] and is widely used (under the comercial name Omadine) as an antifouling agent in various paints, coatings, polymers, and other materials. Pyrithione and its zinc salt are also the active components of antidandruff shampoos and other cosmetics.[26] Thus, *A. stipitatum* is equipped with a very efficient and powerful weaponry to defend itself against attacks of various predators (insect, ruminants, microorganisms etc.). Pyrithione was also shown to effectively inhibit the growth of higher plants.[27] It can be therefore assumed that the ability of *A. stipitatum* to transform 2-PyCNO (**1**) into pyrithione and other pyridine *N*-oxide derivatives represents an important ecological advantage helping the plant to survive in the harsh environment.

Although no studies have been published regarding some adverse effects related to the consumption of *A. stipitatum*, potential health risks should be carefully considered. *N*-Hydroxypyridine-2(1*H*)-thione (pyrithione, **8**), one of the compounds formed from 2-PyCNO (**1**), is known to be very photolabile, giving rise to the hydroxyl radical upon irradiation with UV–visible light (Figure 4).[12,19−21] This reactive radical has been shown to generate modifications of nucleic acid bases and to cause mutations that can initiate carcinogenesis. It also plays an

important role in oxidative damage of cells leading to a number of age-related diseases.[28−30] Furthermore, pyrithione and products of its transformations are suspected of being embryotoxic for fish and other marine organisms and have also been shown to be potentially cytotoxic and genotoxic.[31−34] Thus, it cannot be ruled out that regular consumption of *A. stipitatum* could have some undesirable health consequences. Given the fact that *A. stipitatum* and related species (e.g., *A. rosenbachianum* or *A. suworovii*) are frequently consumed by large populations in Central Asia, it seems to be highly pertinent to evaluate the biological properties of 2-PyCNO-derived compounds in more detail.

■ ASSOCIATED CONTENT

Supporting Information. Selected NMR, IR, UV−vis, and ESI-MS spectra of compounds **1**, **2**, **10**, **11**, **15**, and **16** (NMR and IR spectra of **15** and **16** were recorded using synthetic samples); NI-DART-HRMS and PI-DART-HRMS traces of volatiles formed upon crushing a bulb of *A. stipitatum*; representative HPLC-PDA chromatogram of an ether extract of homogenized bulbs of *A. stipitatum*; ^{13}C NMR data of various *S*-substituted cysteine derivatives; picture of the *Allium stipitatum* plant analyzed; and experimental details about the preparation of compounds **1** and **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

COSY, correlation spectroscopy; DART, direct analysis in real time; DSS, 4,4-dimethyl-4-silapentane-1-sulfonic acid; ESI, electrospray ionization; GC-MS, gas chromatography−mass spectrometry; HETCOR, heteronuclear chemical shift correlation; HMBC, heteronuclear multiple bond correlation; HMDSS, hexamethyldisilane; HPLC, high-performance liquid chromatography; HPLC-MS, high-performance liquid chromatography−mass spectrometry; HRMS, high-resolution mass spectrometry; HSQC, heteronuclear single-quantum correlation; IR, infrared; MCSO, *S*-methylcysteine *S*-oxide (methiin); MTMCSO, *S*-(methylthiomethyl)cysteine 4-oxide (marasmin); NI, negative ionization; NMR, nuclear magnetic resonance; OPA, *ortho*-phthaldialdehyde; PDA, photodiode array; PI, positive ionization; PTFE, polytetrafluorethene; 2-PyCNO, *S*-(2-pyridyl)cysteine *N*-oxide; SPE, solid phase extraction; subg, subgenus; TOF, time-of-flight; UV, ultraviolet.

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