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Nonvolatile *S*-Alk(en)ylthio-L-cysteine Derivatives in Fresh Onion (*Allium cepa* L. Cultivar)

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Supporting Information

ABSTRACT: The L-cysteine derivatives (*R*)-2-amino-3-(methyldisulfanyl)propanoic acid (*S*-methylthio-L-cysteine), (*R*)-2-amino-3-(propyldisulfanyl)propanoic acid (*S*-propylthio-L-cysteine), (*R*)-2-amino-3-(1-propenyldisulfanyl)propanoic acid (*S*-(1-propenylthio)-L-cysteine), and (*R*)-2-amino-3-(2-propenyldisulfanyl)propanoic acid (*S*-allylthio-L-cysteine) were prepared from 3-[(methoxycarbonyl)dithio]-L-alanine, obtained from the reaction of L-cysteine with methoxycarbonylsulfenyl chloride. The occurrence of these *S*-(+)-alk(en)ylthio-L-cysteine derivatives in onion (*Allium cepa* L.) was proven by using UPLC-MS-ESI$^+$ in SRM mode. Their concentrations in fresh onion were estimated to be 0.19 mg/kg *S*-methylthio-L-cysteine, 0.01 mg/kg *S*-propylthio-L-cysteine, and 0.56 mg/kg (*S*-(1-propenyllthio)-L-cysteine, concentrations that are about 3000 times lower than that of isoalliin (*S*-(1-propenyl-*S*-oxo-L-cysteine). These compounds were treated with *Fusobacterium nucleatum*, a microorganism responsible for the formation of mouth malodor. These L-cysteine disulfides were demonstrated to predominantly produce tri- and tetrasulfides. Isoalliin is almost entirely consumed by the plant enzyme alliin lyase (EC 4.4.1.4 *S*-alk(en)yl-*S*-oxo-L-cysteine lyase) in a few seconds, but it is not transformed by *F. nucleatum*. This example of flavor modulation shows that the plant produces different precursors, leading to the formation of the same types of volatile sulfur compounds. Whereas the plant enzyme efficiently transforms *S*-alk(en)yl-*S*-oxo-L-cysteine, mouth bacteria are responsible for the transformation of *S*-alk(en)ylthio-L-cysteine.

KEYWORDS: *Fusobacterium nucleatum*, *Allium cepa* L., alk(en)ylthio-L-cysteine, apotryptophanase

INTRODUCTION

A book published in 2010, *Garlic and Other Alliums: The Lore and the Science*, written by Dr. E. Block, summarizes more than 100 of fascinating discoveries about the chemistry of sulfur compounds originating from odorless *S*-alk(en)yl-*S*-oxo-L-cysteines **1**−**4** (Figure 1).[1] The transformation of these compounds is catalyzed by enzymes called alliinases (EC 4.4.1.4) to yield volatile thiosulfinates (alk(en)yl-*S*(*O*)-*S*-alk(en)yl) via a complex route that has been described in detail and recently reviewed.[1] Alliinases, pyridoxal-5-phosphate-dependent enzymes, are able to polarize, in their active sites, the S(═O) bonds of *S*-alk(en)yl-*S*-oxo-L-cysteines[2] and subsequently to cleave the adjacent bond C—S(O)R to generate unstable sulfenic acids (RS(O)H), which are transformed into thiosulfinates (R—S—S(O)R). Thiosulfinates are inherently reactive and consequently rapidly converted to a variety of di(tri, poly) sulfides. Thiosulfinates have a significant antibacterial activity, apparently because they can react with free RSH or cysteine residues to give *S*-alk(en)ylthio-L-cysteine derivatives. This was observed for the first time by Cavallito et al. in a model system in 1944.[3] We could consequently expect to observe the natural occurrence of *S*-methylthio-L-cysteine **5**, *S*-propylthio-L-cysteine **6**, and *S*-(1-propenylthio)-L-cysteine **7** in *Allium cepa* L. The occurrence of **6** was described for the first time by Dini et al. in 2008.[4] Dini et al.'s paper focused on the antioxidant properties of *A. cepa* L. var. *tropeana* (red onion), but did not explain its isolation and structure in detail. The health benefits and the syntheses of *S*-alk(en)ylthio-L-cysteines **5**, **6**, and **8** were also discussed recently by Zhang et al.[5] These authors explained that **5**, **6**, and **8** are formed from thiosulfinates such as (1-propenyl)1-propenethiosulfinate, an important chemical found in fresh onion tissue macerates,[6] but they did not describe the natural occurrence of **7** in onion or in any *Allium* species.

The flavor industry is interested in creating flavors with more authenticity; therefore, an understanding of the natural systems that modulate flavor perception is of great interest. We demonstrated that mouth bacteria can transform L-cysteine derivatives **9**−**12** into volatile sulfur-containing odorant compounds.[7] We recently proved the natural occurrence of the precursor of 2-methyl-3-sulfanylpentan-1-ol, a key odorant for fresh onion flavor,[8] and during this investigation,[9] by using ultraperformance liquid chromatography−mass spectrometry−electrospray ionization in positive mode (UPLC-MS-ESI$^+$), we generated an unexpected mixture of volatile polysulfur compounds from a specific fraction. This paper describes the discovery of the natural occurrence of **5**−**7** (Figure 1) in fresh and processed onion (*A. cepa* L.) and discusses the in vitro transformation into mixed volatile polysulfides of cysteine derivatives by *Fusobactrium nucleatum*, a microorganism present in oral microflora that is strongly related to the formation of oral malodor.[10−14]

MATERIALS AND METHODS

Materials. All chemical reagents were purchased from Fluka-Sigma-Aldrich (Buchs, Switzerland), Novabiochem (Darmstadt, Germany),

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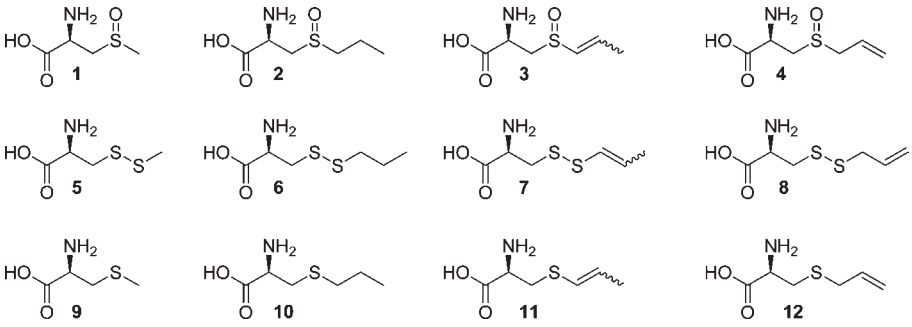

Figure 1. Chemical structures of cysteine conjugates. Names listed in order: *S*-methyl-*S*-oxo-L-cysteine, *S*-propyl-*S*-oxo-L-cysteine, *S*-(1-propenyl)-*S*-oxo-L-cysteine, *S*-allyl-*S*-oxo-L-cysteine, *S*-methylthio-L-cysteine, *S*-propylthio-L-cysteine, *S*-(1-propenylthio)-L-cysteine, *S*-allylthio-L-cysteine, *S*-methyl-L-cysteine, S-propyl-L-cysteine, *S*-(1-propen)-1-yl)-L-cysteine, *S*-allyl-L-cysteine.

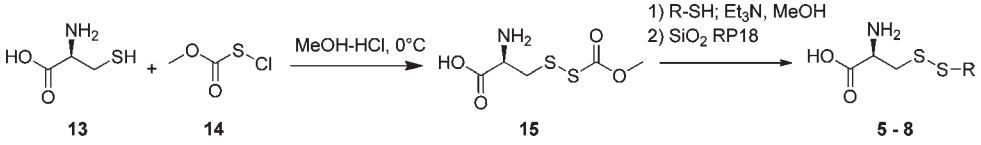

Figure 2. Synthesis of *S*-alk(en)ylthiocysteine derivatives.

SDS Carlo Erba Reactifs (Val-de-Reuil, France), and Acros (Geel, Belgium) unless otherwise specified. The ion-exchange resin was a Dowex 50WX8 200−400 mesh (H^+) (catalog no. L13922) from Alfa Aesar GmbH & Co. (Karlsruhe, Germany), and the reverse phase column was prepared with LiChroprep* RP-18 (40−63 μm, catalog no. 1.13900.0250) from Merck KGaG (Darmstadt, Germany). Samples were filtered prior to injection on UPLC with Acrodisc LC 13 mm syringe filters (0.45 μm, polyvinyledendifluoride membrane) from Pall (New York, NY). The water-soluble internal standard (IS) (2*R*)-2-amino-3-(1-hydroxyheptan-3-ylthio)propanoic acid [$C_{10}H_{21}NO_3S$, molecular weight (MW) 235] was prepared from (2*E*)-heptenal.[9] The volatile IS dodecane was from Fluka-Sigma-Aldrich. *A. cepa* L. cultivar GV120 was purchased from Stoll Frères (Yverdon-les-Bains, Switzerland). The onion powder was a commercial product, Firmenich lot 2009-967746.

^{1}H and ^{13}C NMR Spectra. The NMR spectra were recorded on a Bruker Avance-500 spectrometer (Zurich, Switzerland) at 500.13 and 125.76 MHz. The solvent was 0.01 M DCl. δ values are in parts per million downfield from sodium 3-(trimethylsilyl)tetradeuteriopropionate used to calibrate the chemical shifts (= 0 ppm). The assignments by correlated spectroscopy, heteronuclear single-quantum coherence, and heteronuclear multiple-bond coherence experiments were performed with standard Bruker software (XWINNMR 3.1).

Gas Chromatography (GC)−Electron Impact−MS. An Agilent GC-6890 system connected to an Agilent MSD-5973 quadrupole mass spectrometer (Santa Clara, CA) was operated at ca. 70 eV. Helium was the carrier gas set at a constant flow rate of 0.7 mL/min. Separations were performed on fused-silica capillary columns, coated with SPB-1 (Supelco, Buchs, Switzerland; 30 m × 0.25 mm i.d., 0.25 μm). The standard oven program was as follows: 50 °C for 5 min, increased to 240 °C at 5 °C/min, and then held at 240 °C. MS interpretation was based on the Firmenich mass spectrum database library built from synthetic authentic samples.

UPLC-MS. Analyses were performed on a Waters Acquity system (Waters, Baden-Dättwil, Switzerland) coupled to a mass spectrometer. The separations and quantifications were performed on an Acquity BEH-C18 column (2.1 mm i.d. × 100 mm, 1.7 μm or 2.1 mm i.d. × 150 mm, 1.7 μm). The elution solvents were CH_3CN containing 0.1% formic acid (solvent B) and water containing 0.1% formic acid (solvent A). The gradient profile started at 10% B, was held for 0.5 min, and was then increased to 90% B in 8.0 min. The flow rate was 0.3 mL/min. The separations were also performed on an Acquity BEH-HILIC column (2.1 mm i.d. × 150 mm, 1.7 μm). The elution solvents were CH_3CN containing 0.1% formic acid (solvent B) and water containing 0.1% formic acid (solvent A). The gradient profile started at 5% A, was held for 0.5 min, and was then increased to 50% B in 8.0 min. The flow rate was 0.3 mL/min.

The mass spectrometer was a Thermo Finnigan LXQ (Thermofisher, Basel, Switzerland) with ESI^+. The spray voltage was 4.0 kV. The capillary temperature was 325 °C. The sheath gas was nitrogen at a flow rate of 50 (Finnigan arbitrary units). The auxiliary gas was also nitrogen at a flow rate of 5 (Finnigan arbitrary units). The authentic samples were injected first in full MS mode and then in MSMS mode, with wide band activation at 35 eV.

High-Performance Liquid Chromatography (HPLC) Time-of-Flight (TOF) High-Resolution Mass Spectrometry (HRMS). Analyses were performed on an Agilent 1200 HPLC system composed of a binary solvent manager (pump G1312b). The separation was performed on a Zorbax Eclipse C18 Plus column (Agilent 959764-902; 2.1 mm i.d. × 100 mm, 1.8 μm), thermostated in a column compartment (G1316b) at 60 °C. The mass spectrometry system was an Agilent G1969A TOF MS system composed of a multimode source G1978a (atmospheric pressure chemical ionization + ESI). The solvents used for the separations were water premixed with 0.1% formic acid (Biosolve 23244102 ULC/MSD) (solvent A) and acetonitrile premixed with 0.1% formic acid (Biosolve 01934102 ULC/MSD) (solvent B). The gradient profile was started at 100% A, held for 1 min, and then increased to 100% B in 8 min. The flow rate was 0.5 mL/min, and the injection volume was 1 μL with a G1367C autosampler. A blank run was performed between each sample. The high-resolution TOF had an accuracy of 5 ppm. The fragmentor was set at 140. The scan range was set to 103−1100 by using the online standard for mass adjustment.

GCT TOF HRMS. Analyses were performed on a GCT Premier (Waters, Milford, MA) with an SPB-1 column (30 m, 0.25 mm i.d., 1.0 μm film; Supelco, Sigma-Aldrich Chemie GmbH, Buchs, Switzerland): oven, 60 °C, 5 min, 5 °C/min to 250 °C; constant He flow, 1.0 mL/min, 1.0 μL injected; injector, 250 °C; split, 1:50; solvent delay, 3 min. The acquisition time was set to 0.50 s with an interscan delay of 0.01 s over a

mass range of 1−300 Da. Spectra were recorded using an electron energy of 70 eV, emission current of 2.4 μA, trap current of 200 μA, and source temperature of 200 °C. Calibration was performed using heptacosa (perfluorotributylamine, mass spectrometry grade, Apollo Scientific LTD, Bradbury, U.K.). Calibration data were collected for 1 min in centroid mode. A total of 60 spectra were summed to generate a 21-point calibration curve from m/z 69 to 614 Da. The curve was fitted to a second-order polynomial such that the standard deviations of the residuals were 0.001 amu or lower. Heptacosa was continuously introduced into the ion source, and the ion m/z 218.9856 was used as a lock mass. The mass spectra and molecular formulas were obtained using MassLynx software (Waters). The difference, d, between the exact mass calculated from the molecular formula and that measured was calculated by the software and expressed in ppm [$d = (M_{measd} - M_{calcd})/M_{calcd} \times 106$].

Analysis of Fresh Onion (*A. cepa* L. Cultivar GV120). *Enzymatic Activity Inhibited.* Fresh onions (100 g) were frozen in liquid N_2 and then pulverized with a food processor in the presence of ethanol (EtOH, 50 mL), formic acid (HCOOH, 1 mL), water (50 mL), and a nonvolatile IS [(2*R*)-2-amino-3-(1-hydroxyheptan-3-ylthio)propanoic acid].[9] To check its recovery, 0.1 mg (0.1 mL of 1 g/L standard solution) was added. The suspension was then centrifuged at 8250*g* for 45 min at 5 °C. The liquid phase (130 mL) was used for analysis (Figure 3).

Enzymatic Activity Not Controlled. Fresh onions (100 g) were cut in a food processor (KitchenAid) in the presence of water (100 mL), and 0.1 mg of the IS was added. The suspension was then centrifuged at 8250*g* for 45 min at 5 °C. Formic acid (1 mL) was added to the liquid phase (130 mL) before analysis.

Analysis of an Industrial Quality Onion Powder. The authentic samples were obtained in their neutral forms, and their purities were >95% by ^{1}H NMR. Calibration curves were established by using two stock solutions, and dilutions were made to obtain concentrations of 0.1, 0.5, 1.0, 5.0, and 20 μg/mL. The calibration solutions were injected before and after the samples were subjected to analysis in SRM mode. The values for the square of the Pearson correlation coefficient (rsq) were always between 0.98 and 0.99; the quantitative values given below correspond to the mean value of the concentration, the values in parentheses corresponding to standard deviations.

The sample preparation was as straightforward as possible; specifically, 50 g of onion powder was suspended in 505 mL of water, ethanol, and formic acid, and a nonvolatile IS was added. The solid was removed by centrifugation, and the liquid was filtered on a 0.45 μm membrane. The purpose of adding an IS was to determine a recovery factor. The IS solutions used to establish the calibration curve were stable at 4 °C for >1 week.

Preparation of 3-[(Methoxycarbonyl)dithio]-L-alanine 15 (Figure 2).[15] To methoxycarbonylsulfenic chloride **14** (1 mL, 11 mmol) in methanol (8 mL) at 0 °C was added a solution of (L)-cysteine−HCl **13** (890 mg, 5.6 mmol) in methanol−HCl (7 mL, 1.25 M, Fluka) over 20 min. The solution was stirred for 1 h, and then the excess reagent and methanol were removed under vacuum.

Preparation of *S*-Methylthio-L-cysteine 5. The 3-[(methoxycarbonyl)dithio]-L-alanine **15** (6.9 g, 28 mmol) in anhydrous methanol (50 mL) was added to methanethiol (5.29 g, 110 mmol) and triethylamine (3.5 mL, 25 mmol) in methanol (50 mL). The pH was maintained between 8 and 9 by adding triethylamine (3 × 3 mL), and then the reaction was stirred overnight. The resulting mixture was diluted with water (100 mL), and the organic compounds were extracted with pentane. The water phase was loaded on a SiO_2-RP18 column (i.d. 6.5 cm, level of dry phase 10 cm), and the column was eluted with water. Fractions containing the compound were lyophilized; we obtained 600 mg. The proton NMR indicated the presence of triethylammonium salts and the formation of a methyl ester of cysteine, the major side product. Product **5** was further purified on a Dowex column 50WX8, with gradient elution of aqueous ammonia at 0, 0.3, 0.6, 0.9, 1.2, 1.5, 1.8, 2.1, and 2.3 M. Compound **5** was eluted in the fraction eluted with 1.8 M ammonia. We obtained 280 mg (yield 30%): HPLC-TOF HRMS, $C_4H_{10}NO_2S_2$, calculated mass 167.00747, measured 167.00849 (difference 6.1 ppm); HPLC-ESI$^+$ (BEH-C18, 150), 1.18 min (MW + H) 168, for SRM fragments used 79 (100%); ^{13}C NMR, δ 24.4 (q), 39.8 (t), 56.2 (d), 175.6 (s) (5) (see the Supporting Information).

Preparation of *S*-Propylthio-L-cysteine 6. 3-[(Methoxycarbonyl)-dithio]-L-alanine **15** (1.39 g, 5.6 mmol) in anhydrous methanol (10 mL) was added to 1-propanethiol (2 mL, 22 mmol), triethylamine (0.7 mL, 5 mmol), and methanol (10 mL). The pH was maintained between 8 and 9 by adding triethylamine (3 × 0.3 mL), and then the reaction was stirred overnight. The resulting mixture was diluted with water (100 mL), and the organic compounds were extracted with pentane. The water phase was loaded on a SiO_2-RP18 column (i.d. 4.5 cm, level of dry phase 10 cm). The column was rinsed with water (400 mL) and with water with 10, 15, or 20% EtOH (200 mL), and the product was eluted with water containing 30% EtOH (500 mL). After lyophilization, **6** was obtained (750 mg, yield 69%): HPLC-TOF HRMS, $C_6H_{14}NO_2S_2$, calculated mass 195.03877, measured 195.03709 (difference 8.6 ppm); HPLC-ESI$^+$ (BEH-C18, 150), 4.61 min (MW + H) 196; for SRM fragments used, 107 (100%); ^{13}C NMR, δ 15.2 (q), 24.6 (t), 39.6 (t), 42.7 (t), 54.6 (d), 173.4 (s) (5) (see the Supporting Information).

Preparation of *S*-Allylthio-L-cysteine 8. 3-[(Methoxycarbonyl)-dithio]-L-alanine **15** (72.4 g, 16.9 mmol) in anhydrous methanol (30 mL) was added to 2-propenethiol (5.37 g, 74 mmol), triethylamine (2.61 g, 25.8 mmol), and methanol (30 mL). The pH was maintained between 8 and 9 by adding triethylamine (3 × 0.3 mL), and then the reaction was stirred overnight. The resulting mixture was diluted with water (100 mL), and the organic compounds were extracted with pentane. The water phase was loaded on a SiO_2-RP18 column (i.d. 4.5 cm, level of dry phase 10 cm). The column was rinsed with water (400 mL), and then the product was eluted with water containing 10, 20, 30, or 40% EtOH (200 mL). Compound **8** was eluted with 30% aqueous EtOH. After removal of the ethanol, lyophilized **8** was obtained (1.6 g, yield 49%): HPLC-TOF HRMS, $C_6H_{12}NO_2S_2$, calculated mass 193.0228, measured 193.02312 (difference 1.6 ppm); HPLC-ESI$^+$ (BEH-C18, 150), 2.70 min (M + 1) 194; for SRM fragments used, 152 (100%), 105 (30%); ^{13}C NMR, δ 39.7 (t), 43.5 (t), 54.6 (d), 122.2 (t), 136.2 (d), 173.6 (s) (5,*S*).

Preparation of 3 *S*-(1-Propenylthio)-L-cysteine. (*E*/*Z*)-1-Propenethiol was obtained after reduction of bis[(*E*/*Z*)-1-propenyl]disulfide (0.9 g, 6 mmol)[16] with $LiAlH_4$ (0.24 g, 6 mmol) in diethyl ether (Et_2O) (20 mL) overnight at room temperature. Salts and excess $LiAlH_4$ were removed after careful addition of ice water and HCl (2 M). The organic phase was separated and dried on Na_2SO_4. The volume of Et_2O was reduced to 5 mL, and the solution was added to 3-[(methoxycarbonyl)dithio]-L-alanine **15** (5.5 mmol) in anhydrous methanol (10 mL). Triethylamine was added (0.7 mL, 5 mmol) until the pH was 3−4. After 1 h, an additional 0.2 mL of triethylamine was added until the pH was 6−7, and then the reaction was stirred overnight. The resulting mixture was diluted with water (100 mL), and the organic compounds were extracted with pentane. The water phase was loaded on a SiO_2-RP18 column (internal diameter 4.5 cm, level of dry phase 10 cm). The column was rinsed with water (400 mL) and then water with 10% EtOH (200 mL) and water with 20% EtOH (200 mL), and the product was eluted with aqueous EtOH 30% (400 mL). After the material was lyophilized, we obtained 400 mg of a white powder, which was further purified by chromatography under the same conditions; only the purest fraction, containing **7**, was retained (60 mg, yield 9%): HPLC-TOF HRMS, $C_6H_{12}NO_2S_2$, calculated mass 193.02101, measured 193.02312 (difference 10.9 ppm); HPLC-ESI$^+$ (BEH-C18, 150), 4.48 min (M+1) 194; for SRM fragments used, 105 (100%), 131 (40%). No separation of (*E*/*Z*) isomers. 7-(*Z*): ^{1}H NMR, δ 6.19 (dq, J = 9.1, 1.6 Hz, 1H, H-4), 5.96 (dq, J = 9.1, 6.9 Hz, 1H, H-5), 4.26−4.21 (m, 1H, H-2), 3.34 (dd,

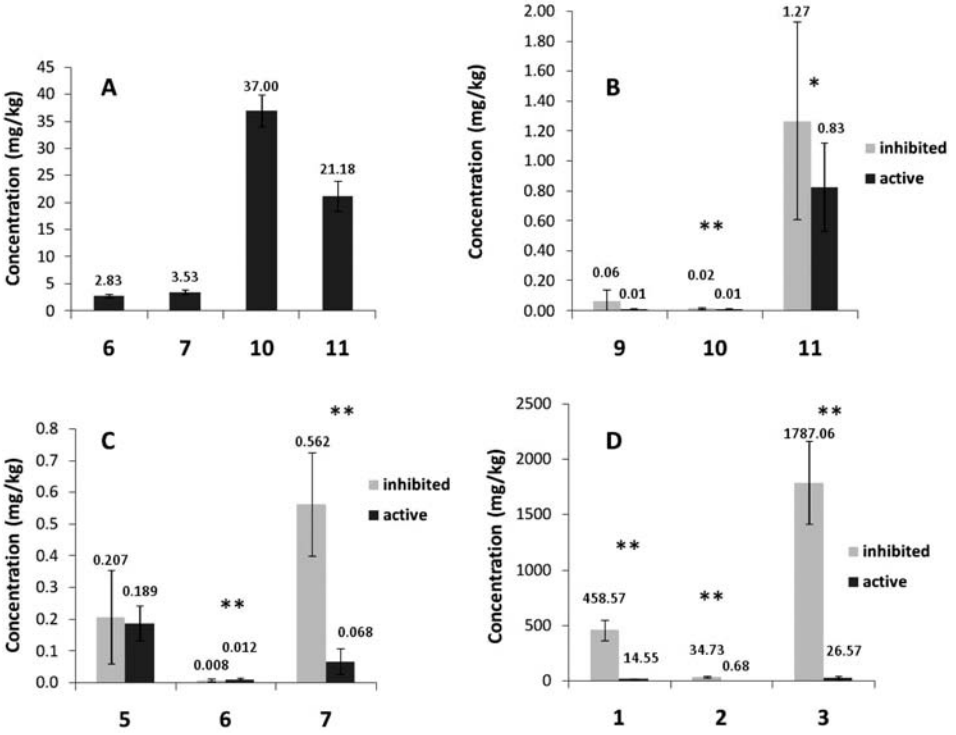

Figure 3. Concentrations of compounds **1**−**3**, **5**−**7**, and **9**−**11** in onions (mean ± 95% confidence interval): (A) concentrations of **6**, **7**, **10**, and **11** in onion powder; (B) concentrations of sulfides **9**−**11** in fresh onion (fresh onions were pulverized when frozen with liquid N_2; enzymatic activity was inhibited with EtOH/HCOOH; otherwise, it was labeled as "active"); (C) concentrations of disulfides **5**−**7** in fresh onion; (D) concentrations of sulfoxides **1**−**3** in fresh onion. ∗, differences are significant when $p < 0.05$ (∗∗, 95% confidence level; ∗, 90% confidence level).

J = 15.1, 4.0 Hz, 1H, H-3), 3.16 (dd, J = 15.1, 8.2 Hz, 1H, H-3′), 1.77 (dd, J = 6.9, 1.6 Hz, 3H, H-6); ^{13}C NMR, δ 174.7 (s), 134.5 (d), 129.0 (d), 55.4 (d), 39.6 (t), 16.8 (q). 7-(*E*): ^{1}H NMR, δ 6.17−6.14 (m, 2H, H-4 and H-5), 4.26−4.21 (m, 1H, H-2), 3.38 (dd, J = 15.1, 4.1 Hz, 1H, H-3), 3.17 (dd, J = 15.1, 7.8 Hz, 1H, H-3′), 1.79 (m, 3H, H-6); ^{13}C NMR, δ 174.6 (s), 137.8 (d), 125.1 (d), 55.5 (d), 40.6 (t), 20.4 (q).

Release of Free Thiols from *S*-Alk(en)ylthio-L-cysteine by Apotryptophanase in a Model System (Figure 5B). The buffer solution was potassium phosphate (100 mM, pH 8) containing ethylenediaminetetraacetic acid (1 mM), pyridoxal 5-phosphate (0.1 mM), and L-glutathione (reduced form, 1 mM). Apotryptophanase (from *Escherichia coli*, activity 75−150 units/mg, Sigma-Aldrich) was freshly prepared (1 mg in 0.5 mL of buffer). Standard solutions of authentic samples (0.01 mL, containing 10 μg of each precursor) **5**−**7** were added to the buffer (1 mL) and the enzyme in the buffer (0.5 mL). The mixture was incubated for 30 min at 35 °C in sealed vials. The incubated mixture was cooled in ice and toluene (0.5 mL), which contained IS for GC analysis: dodecane (10 mg/L) and 20 μL of concentrated HCl were added. The solution was vigorously stirred and then centrifuged. We made two blanks: one without the enzyme (Figure 5C) and one without the precursors. The organic phase was injected on GC-MS (Figure 5B), and the aqueous phase was injected on LC-MS.

Release of Free Thiols from *S*-Alk(en)ylthio-L-cysteine by *F. nucleatum* DSM 20482 in a Model System (Figure 5A,D,E). *F. nucleatum* DSM 20482 (3.6×10^7 cfu/mL) in sterile, degassed saline solution (0.9% NaCl) was prepared as described:[7] 2.0 mL was added to 500 μL of buffer phosphate (0.1M, pH 8) containing standard solutions of **1**−**3**, **5**−**7**, or **9**−**11** (0.01 mL, containing 10 μg of each precursor). The mixtures were incubated at 35 °C for 15 h. The organic compounds were extracted as described earlier for the analysis of volatiles by GC-MS and GC-HRMS (Figure 5A,D,E). The water phase (0.1 mL) was diluted in 0.1% aqueous formic acid (0.9 mL) and filtered prior to injection on the UPLC.

Addition of Fresh Onion to 4, 8, or 12 (Figure 6). Fresh onions (100 g) were added to **4**, **8**, or **12** (50 mg each/100 mL of water) and processed in a food processor. The puree was centrifuged at 5 °C for 30 min at 8250*g*. The clear solution was extracted with pentane (2 × 100 mL). The organic phase was dried on Na_2SO_4, filtered, and concentrated to 0.5 mL prior to injection.

RESULTS AND DISCUSSION

Formation of Volatile Polysulfides from an Onion Powder Fraction Treated with a β-Lyase. The analysis of onion powders offers two advantages over fresh onions: the heat treatment used during the drying process stops the enzymatic activity, and large quantities of this industrial product are available. The extraction of onion powder started with the addition of water. The non-water-soluble part of the onion powder was removed by centrifugation. The water-soluble compounds were then separated on a reverse phase HPLC column.[9] The first fractions that eluted with water contained mineral salts; the next fractions, still eluted with water, contained *S*-alk(en)yl-oxo-L-cysteine **1**−**3** and **11**. Then, less polar compounds **10** and **11** (Figure 1) were detected in the following fractions eluted with 10% aqueous EtOH. The fraction obtained with 20% aqueous EtOH was also lyophilized because we reasoned that the precursor of 2-methyl-3-sulfanylpentan-1-ol might be found in this fraction.[9] This last fraction was treated with apotryptophanase, an enzyme having β-lyase activity. The β-lyase needs a free amino functional group; therefore, we can rule out the

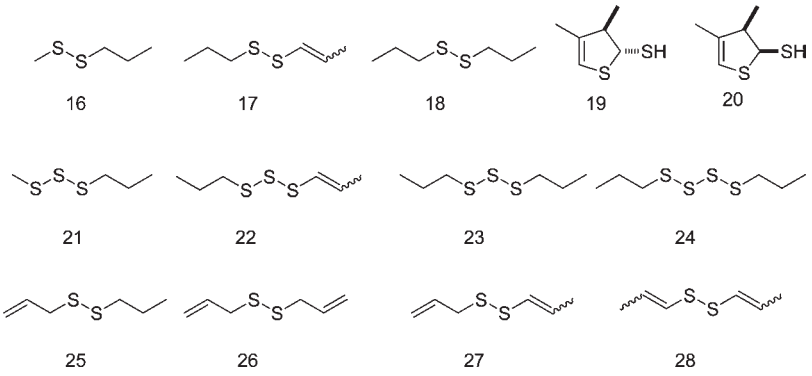

Figure 4. Chemical structures of volatile organic sulfur compounds released by β-lyase activity of alliinase.

involvement of γ-glutamyl derivatives. The organic volatile compounds were extracted with toluene and injected on GC-MS and GC-HRMS TOF, which allowed us to identify sulfides **16**−**24**. In decreasing order of importance, in terms of peak area, we saw two peaks with the same MS for 1-(1-propen-1-yl)-3-propyltrisulfane **22**, a structure suggested by the MS library and confirmed with GC-HRMS, the ^{32}S-**22** HRMS giving $C_6H_{12}S_3$: 180.0117 (calculated 180.0101); dipropyltrisulfane ^{32}S-**23** displayed a base peak of 182.0268 (calculated 182.0258) and a smaller signal for ^{34}S-**23** (MW + 2) = 184, representing the isotopic abundance of ^{34}S. The peak intensity of the ^{34}S isomers represented 12.6% (calculated 13.2%) of the ^{32}S isomers, which confirmed the occurrence of three sulfur atoms. The major MS fragment of **23** was C_3H_7S. The fourth peak in terms of abundance was dipropyltetrasulfane **24**: MW 213.9989, measured as 213.9989 fragmenting into $C_3H_3S_2$ (HRMS 108.0069, calculated 108.0067). The other important peaks were methyl 1-methyl-3-propyltrisulfane **16**, 1-(propyldithio)-1-propene **17**, 1-methyl-3-propyltrisulfane **21**, and both stereoisomers of 3,4-dimethyl-2-sulfanyl-2,3-dihydrothiophene (DMMT), **19** and **20**, which could be GC artifacts of di-1-propen-1-yl disulfide **28**.[16]

These volatile organic polysulfides could not originate from **1**−**3** or **9**−**11** because these precursors were identified in the first fractions of the separation. The most logical hypothesis was that these compounds were originating from *S*-alk(en)ylthio-L-cysteine. The β-lyase cleaves the C−S−S−R bond, releasing a reactive X−S−S−R (X = negative charge or proton), which will react further with other sulfur compounds. Looking at the LC-MS ESI$^+$ trace, it was possible to detect the molecular ions of **5**, **6**, and **7** at M + H = 168, M + H = 196, and M + H = 193, respectively. Without having in hand the authentic samples, it was not possible to confirm this hypothesis.

Preparation of Authentic Samples. To confirm our hypothesis, we prepared authentic samples as described previously in the literature without improvements in the procedure; our goal was to make the purest references possible. The *S*-methyl, *S*-propyl, and *S*-allyl cysteine derivatives **9**, **10**, and **12** were prepared from cysteine **13** in aqueous sodium hydroxide solution containing ethanol by addition of the corresponding alk(en)yl bromides.[17,18] All compounds were purified by ion exchange chromatography, and the purities were checked by NMR and compared with published data.[19−21] Oxidations to prepare **1**, **2**, and **4** were performed with H_2O_2, as described for the first time by Stoll and Seebeck in 1949,[22] and the spectroscopic data were in agreement with more modern publications.[21,23] Isoalliin **3** was obtained after isomerization of *S*-allyl-L-cysteine **12** with potassium *tert*-butoxide in dimethyl sulfoxide to prepare (*E*/*Z*)-**11**, which was then oxidized with H_2O_2 to give a mixture of *R*/*S*/*E*; *R*/*S*/*Z*; *R*/*R*/*E*; *R*/*R*/*Z* **3**.[20−22] The preparation of *S*-alk(en)ylthio-L-cysteines **5**−**8** was more challenging. The first preparation described by Cavallito et al.[3] started with the reaction of thiosulfinates with cysteine, and **8** was obtained and fully characterized.[3,24,25] Recently, Zhang et al.[5] prepared mixed disulfides from dithiophosphoric acids with good yields (70−80%), but they were unable to make compound **7**. Compounds **5**−**8** were prepared from **15**, obtained from the reaction of cysteine **13** with methoxycarbonylsulfenyl chloride **14** (Figure 2). The thiols corresponding to the desired alk(en)yl side chains were added to this activated cysteine **15**.[15] The di-1-propen-1-yl disulfide **28** was prepared via the cleavage of 1-(methylthio)-1-propene with lithium in ammonia, followed by oxidation with I_2, as described by Block and Thiruvazhi.[16] The disulfide **28** was reduced with $LiAlH_4$ just before its addition to the activated cysteine **15**. No optimization was considered; we focused only on having compound (*E*/*Z*)-**7** as pure as possible (Figure 2).

Occurrence of *S*-Alk(en)ylthio-L-cysteine Derivatives 6 and 7 in Onion Powder. We screened for *S*-(propyl disulfanyl)cysteine **6** (M + H = 196, SRM 107) and *S*-(1-propenyl disulfanyl)cysteine **7** (M + H = 194, SRM 132 and 105) in onion powder. The objective of this work was not to precisely quantify the cysteine derivatives, but to understand the trends. The recovery factor, calculated from a nonvolatile IS, was between 70 and 90%. Coeluting matrix components can affect ionization, which may explain differences in the recovery factor values. To overcome this well-documented problem, we could have considered many techniques.[26] No correction factors taking into account the recoveries of the nonvolatile IS were calculated. The focus was on the relative abundances of the precursors.

The extractions were repeated three times, and each extract was injected twice. The results were consistent: we observed 37.0 mg/kg (±3.7) (mean ± SD) and 21.2 mg/kg (±1.1) for **10** and **11**, respectively. Derivatives **6** and **7** were quantified at 2.8 (±0.1) and 3.5 (±0.2%) mg/kg, respectively (Figure 3A). This is the first time, to our knowledge, that the natural occurrence of **7** is described.

Occurrence of Cysteine Conjugates 1−3, 5−7, and 9−11 in Fresh Onion or in Fresh Onion with Inhibited Enzymatic Activity. The analyses were performed with fresh onion juice and also onion juice prepared in the presence of ethanol and formic acid to inhibit or block enzymatic activity. We were unable to find direct quantification of isoallin **3** in previously reported work, as most of the published examples quantified **1**−**3** after derivatization.[1,27]

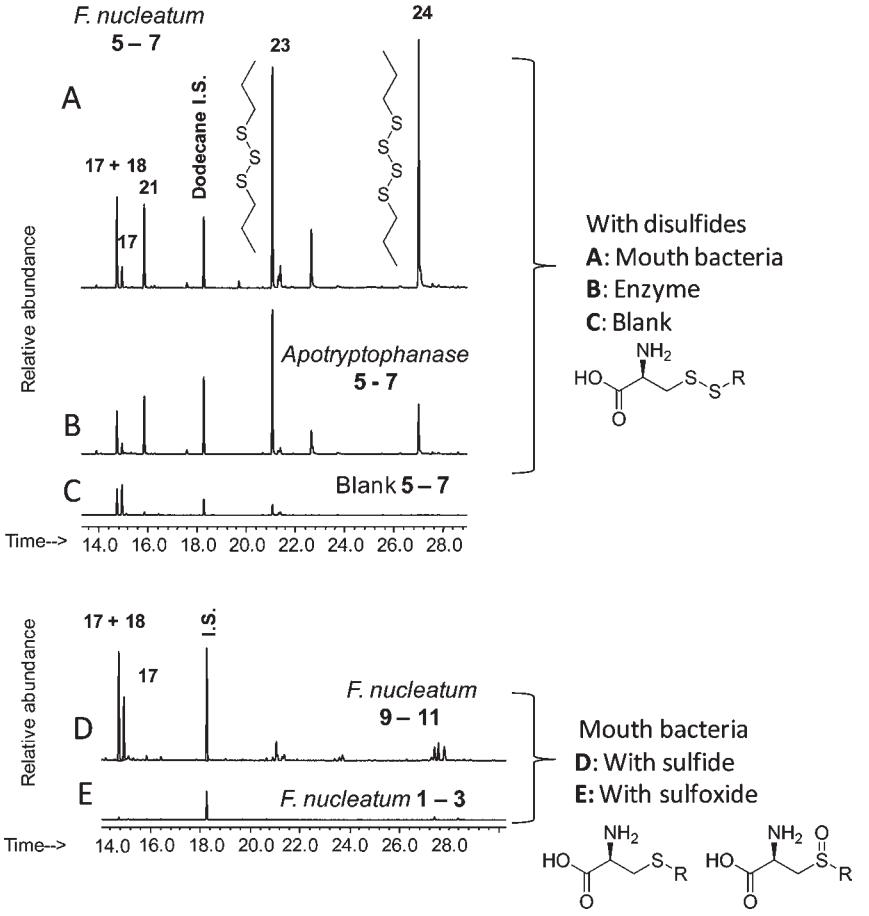

Figure 5. Comparison of GC-MS traces of an organic extract of volatiles produced by *F. nucleatum* and apotryptophanases from various cysteine-$(S)_m(O)_n$-conjugates (m = 1 or 2, n = 0 or 1) at the same concentrations. The IS provided a relative quantitative indication. The blank (C) was incubated for 15 h at pH 8 and 35 °C. (MS available in the Supporting Information.)

Conjugates **5**−**12** were analyzed on a reverse phase Acquity BEH C-18 column, but the more polar sulfoxides **1**−**3** eluted at 0.85, 1.54, and 1.23 min, respectively, coeluting with salts, sugars, and other polar compounds, which interfere with LCMS-ESI$^+$ detection. Therefore, they were analyzed on an Acquity BEH amide column, which gave satisfactory results, and the concentration of **3** in fresh enzymatically inhibited onions was 1787 mg/kg, the mean value of analysis of different onion lots. The lowest value was 1200 mg/kg, and the highest was 2200 mg/kg. These values are consistent with previously published data[27] (Figure 3D). The mean concentration of *S*-methylcysteine **9** in fresh and inhibited onions was 0.06 mg/kg, and the maximum observed in one lot was 0.22 mg/kg. The concentration differences between fresh and inibited onions was not significant. The mean concentration of **10** was 0.02 mg/kg and very consistent between lots. In inhibited onions, the concentration of *S*-(1-propen-1-yl)-L-cysteine**11** was 1.27 mg/kg, and in fresh noninhibited onion, it was 0.83 mg/kg (Figure 3B).

The quantitative analysis of *S*-alk(en)ylthio-L-cysteine disulfides was performed on six types of Swiss onion; four of them were replicated. We consequently obtained 10 values for the inhibited series and 10 values for the fresh onion series. The mean quantity for **5** in nonblocked fresh onion was 0.19 mg/kg; the maximum value measured for one lot was 0.34 mg/kg, and these concentrations were quite stable over different lots. In the inhibited series, **5** was 0.21 mg/kg. These results indicate that there is no significant difference between inhibited and fresh onion preparations in the concentrations of **5**. The cysteine derivative **6** can be detected; it was in fresh and inhibited onions at 0.01 mg/kg, and the highest value observed was 0.02 mg/kg (Figure 3C). This compound is a minor substance compared with **7**, which was 50 times more abundant (0.56 mg/kg). The concentration of compound **7** decreased significantly when the enzymatic activity was not blocked (0.07 mg/kg).

Model Enzymatic Transformation System of Cysteine Conjugates 1−3, 5−7, and 9−11. As is well documented and understood, onion enzymes are very efficient at transforming cysteine-$S(O)$-conjugates **1**−**3** into volatile organic sulfur compounds.[1,28] The volatile organic compounds formed from nonvolatile precursors and discussed in this work are listed in Figure 4 It is has not been well documented that cysteine-S_n-conjugates such as **5**, **6**, and **9**−**11** (Figure 3B,C) are little affected by onion enzymes, even if the result for compound **7** (Figure 3C) is misleading. Compound **7** is not stable, as illustrated in the GC-MS trace of the blank (Figure 5C), but it is stable for more than 15 h in acidic solutions used for calibrations. We assumed that the concentration difference of **7** in fresh or inhibited onion (Figure 3C) is not associated with direct enzymatic activity.

To better understand and confirm these observations, we performed several experiments. The first experiment was to add *F. nucleatum* to *S*-alk(en)ylthio-L-cysteine **5**−**7** (Figure 5A) (*S*).

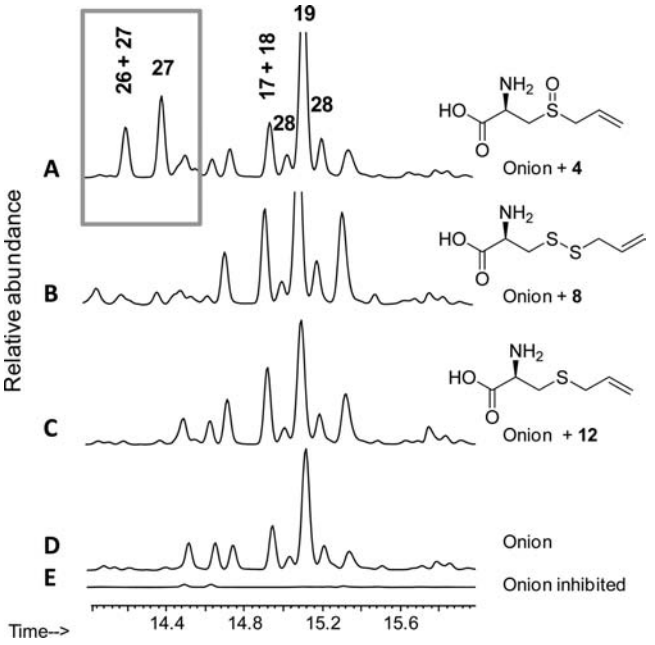

Figure 6. Relative peak area comparison of GC-MS traces of an organic extract with active onion enzymes alone and with the addition of precursors **4** (alliin), **8**, or **12**, which do not naturally occur in significant amounts in onions. The lower trace is of inactivated onion enzymes (= blank). (Stereochemistry of *Z*/*Z*; *E*/*Z*; *E*/*E* **28** not specified.)

F. nucleatum is a Gram-negative anaerobic rod microorganism present in the human mouth. It is a relevant model for the generation of mouth malodor[7,10−14] and accepted as being an important contributor to halitosis because it can ferment peptides to generate hydrogen sulfide and methyl mercaptan.[29−33] In this experiment, we followed the consumption of precursors by LC-MS and monitored the generation of volatile organic compounds by GC-MS of organic extracts. The reaction mixture was extracted with toluene containing 10 mg/L of dodecane as a volatile IS. The major volatiles produced were **23** and **24**. Over three repetitions, the results were consistent, and only the ratio of **23** to **24** changed slightly. This experiment was repeated with apotryptophanase, a commercially available, easy-to-handle enzyme having β-lyase activity.[34] The transformations of **5**−**7** were performed in a phosphate buffer. Figure 5B displays a similar pattern formation of volatiles containing tri- and tetrasulfides **23** and **24**. Compounds **5**−**7** were not stable at 35 °C, pH 8, for 15 h (Figure 5C), and the decomposition resulted in the formation of disulfides. Figure 5D displays the volatiles observed by fermentation of *F. nucleatum* with **9**−**11**. The transformation of cysteine-*S*-conjugates produced the alk(en)yl mercaptans, which were detected before the toluene peak eluted by GC-MS (not shown), disulfides **17**, and **18**, and no polysulfides. When **1**−**3**, in equivalent quantities, were treated with *F. nucleatum* in replicate trials, they were not transformed (Figure 5E) and were more stable than *S*-alk(en)ylthio-L-cysteine and *S*-alk(en)yl-L-cysteine.

We expected to see more reaction products of **7** in comparison to **23** and **24**. The lack of these reaction products may be due to the stability and reactivity of 1-propenyl−S−H, which is probably less favorable than propyl−S−SH in forming polysulfides.

The last experiment was performed to understand whether the onion enzymes can transform cysteine derivatives in volatile organic sulfur compounds. It has already been proven that **9** is not transformed with isolated allinase isoforms I and II, but not yet for the entire enzyme system in onions.[35] Fresh onions were frozen and then ground into powder. When the enzymatic activity was blocked and the onion warmed, no sulfur compounds were formed (Figure 6D). If the enzymatic activity was not inhibited, when the frozen onion was warmed, we could observe the formation of dipropenyl disulfide **28** and of 1-propenyl derivatives such as **17** and **19** (**20** and **22**, not shown) (Figure 6C), in agreement with the abundance of isoalliin **3** (1−2 g/kg). An aqueous solution containing precursors **5**−**7** and **9**−**11** (0.25 g/kg each) was added to the still-frozen onion powder, but after warming it, we did not observe a significant difference compared with the onion puree (Figure 6C). This observation indicates that onion enzymes cannot transform nonoxygenated sulfur derivatives, but stronger evidence was required. An alternative route to show that the onion enzymes cannot degrade cysteine-*S*-conjugates was the addition of 0.250 g/kg of alliin **4** to 100 g of fresh onion (Figure 6A). Alliin is naturally abundant in garlic, but occurs in onion at very low concentrations. The same experiment was repeated with **8** and **12** (Figure 6B,C). If onion alliinase is efficient only with sulfoxides, volatile organic compounds with allyl moieties should be formed on the addition of **4** but not with the nonoxygenated derivatives **8** and **12**. This is the result that we observed, shown in Figure 6A−C, at 14.21 and 14.39 min, with **26** and **27**. This finding confirmed that onion enzymes preferentially transform sulfoxides over sulfide derivatives.

All conjugates were tasted in a water solution at 1 mg/kg in the same conditions as described earlier.[7] From this tasting, we noted that **9** released a rubbery, sulfury onion odor, with the same type of delayed effect as described previously for **10**.[7] Compound **7** developed a fried onion odor in the mouth, similar to 3,4-dimethyl-2,3-dihydrothiophene-2-thiol and slightly different from **11**, but no detailed sensory analyses were performed. No special odor was perceived when compounds **2** and **4** were tasted at 1 mg/kg, in agreement with the results discussed earlier. We can speculate that **5**−**7** and **9**−**11** have an impact on the odor perception of onion flavor resulting from the action of β-lyases in the mouth,[7,36−40] whereas **1**−**3**, if not totally transformed by alliinase when the onions are chopped, are not transformed by mouth bacteria and so will not develop a sulfury odor.

From this study, we cannot demonstrate the true flavor impact of the presence of **5**−**7** and **9**−**11** when we eat a fresh onion. The massive amount of **1** and **3** transformed in volatile sulfur compounds most probably masks the impact of volatiles released by mouth bacteria from **5**−**7** and **9**−**11.** In theory, however, we can speculate that the amount of **7** and **11** is sufficient to have a flavor impact (0.6 and 1.3 mg/kg for **7** and **11**, respectively) because the odor threshold of these sulfur compounds is less than a few micrograms per kilogram.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. Further details of compounds **5**−**8**. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

GC, gas chromatography; HPLC, high-performance liquid chromatography; IS, internal standard; HRMS, high-resolution mass spectrometry; MW, molecular weight; TOF, time-of-flight; UPLC-MS-ESI$^+$, ultraperformance liquid chromatography−mass spectrometry−electrospray ionization in positive mode; SRM, selected reaction monitoring.

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