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Applications of Direct Analysis in Real Time−Mass Spectrometry (DART-MS) in *Allium* Chemistry. (*Z*)-Butanethial *S*-Oxide and 1-Butenyl Thiosulfinates and Their *S*-(*E*)-1-Butenylcysteine *S*-Oxide Precursor from *Allium siculum*

Roman Kubec,*,† Robert B. Cody,*,‡ A. John Dane,‡ Rabi A. Musah,#
Jan Schraml,§ Abith Vattekkatte,# and Eric Block*,#

†Department of Applied Chemistry, University of South Bohemia, Branišovská 31, 370 05 České Budějovice, Czech Republic, ‡JEOL USA, Inc., 11 Dearborn Road, Peabody, Massachusetts 01960, #Department of Chemistry, University at Albany, SUNY, Albany, New York 12222, and §Institute of Chemical Process Fundamentals of the AS CR, Rozvojová 135, 165 02 Prague 6, Czech Republic

Lachrymatory (*Z*)-butanethial *S*-oxide along with several 1-butenyl thiosulfinates was detected by DART mass spectrometry upon cutting *Allium siculum*, a popular ornamental *Allium* species used in some cultures as a spice. (*Z*)-Butanethial *S*-oxide isolated from the plant was shown to be identical to a synthetic sample. Its likely precursor, (R_S,R_C,*E*)-*S*-(1-butenyl)cysteine *S*-oxide (homoisoalliin), was isolated from homogenates of *A. siculum*, and a closely related species *Allium tripedale*, and fully characterized. Through use of LC-MS, a series of related γ-glutamyl derivatives were tentatively identified in *A. siculum* and *A. tripedale* homogenates, including γ-glutamyl-(*E*)-*S*-(1-butenyl)cysteine and its *S*-oxide, γ-glutamyl-*S*-butylcysteine and its *S*-oxide, and γ-glutamyl-*S*-methylcysteine and its *S*-oxide. Because compounds containing the 1-butenyl group have not been previously identified in genus *Allium* species, this work extends the range of known *Allium* sulfur compounds. The general applicability of DART mass spectrometry in identifying naturally occurring, thermally fragile thial *S*-oxides and thiosulfinates is illustrated with onion, *Allium cepa*, as well as a plant from a different genus, *Petiveria alliacea*.

KEYWORDS: *Allium siculum*; *Nectaroscordum*; *Allium cepa*; *Petiveria alliacea*; DART mass spectrometry; butanethial *S*-oxide; *S*-(1-butenyl)cysteine *S*-oxide; structure elucidation; homoisoalliin; lachrymatory sulfines

INTRODUCTION

There is considerable interest in understanding how plants use secondary metabolites to defend themselves against predator attack. Onion, garlic, and other genus *Allium* plants store defense-related secondary metabolites as inactive precursor *S*-alk(en)ylcysteine *S*-oxides, which are converted into unstable chemically and biologically active agents by plant enzymes in response to a challenge (*1*−*3*). Upon tissue damage caused by pest attack, comingling of the normally separated enzyme and precursors occurs, resulting in a cascade of chemical reactions releasing a burst of defensive compounds. These electrophilic compounds, which can be difficult to detect due to their fleeting existence and thermal sensitivity, are postulated to produce pain, lachrymation, and inflammation in animal predators by activating TRPA1 and TRPV1, excitatory ion channels on primary sensory neurons of the pain pathway, to achieve deterrence (*4*−*6*).

Allium siculum (**Figure 1**, top), is a prime example of a plant generating a diversity of irritant defensive compounds, for example, both a lachrymatory thial *S*-oxide and vesicant thiosulfinates. We use a powerful new analytical technique, direct analysis in real time mass spectrometry (DART-MS; **Figure 1**, bottom), to very simply and directly identify these defensive agents from fresh plant samples at room temperature, without the use of solvents or processing. Furthermore, we have isolated and fully characterized from *A. siculum* as well as from a closely related species, *Allium tripedale*, the stable cysteine-based precursors of these compounds. Our work continues earlier studies with *A. siculum* (*7*) and significantly extends the range of organosulfur precursors and odor and flavor compounds found in genus *Allium* plants. It is notable that Finnish Nobel Laureate Artturi Virtanen, who studied the onion lachrymatory factor (LF) more than 40 years ago, anticipated the types of compounds we found in *A. siculum* (*8*). To put our work in perspective, we discuss the plant *A. siculum*, summarize the organosulfur chemistry (**Figure 2**) of *Allium cepa* (onion) and a plant from a different genus, *Petiveria alliacea* (Phytolaccaceae), as excellent models for *A. siculum* chemistry, and discuss DART-MS, which provided the key to our work.

A. siculum, an attractive ornamental bulbous plant, is a member of a small subgenus *Nectaroscordum* of the genus *Allium*, consisting of only this species and *A. tripedale* (*9*), the latter being

*Corresponding authors [(R.K.) telephone +420 387 772 664, fax +420 385 310 405, e-mail kubecr@centrum.cz; (R.B.C.) telephone (978) 535-2396, fax (978) 536-2205, e-mail cody@jeol.com; (E.B.) telephone (518) 442-4459, e-mail eb801@albany.edu].

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Figure 1. (Top) *A. siculum* flowers (left) and foliage (right). (Bottom) DART instrument showing autosampler (left) and sample positioned in DART probe (right).

a rare plant indigenous to Armenia, Iran, and Iraq, where it is used in folk medicine. *A. siculum* (Sicilian honey lily, Sicilian honey garlic, or Mediterranean bells), is native to Asia Minor, southern France, and Sicily, growing in damp, shady woods. It has showy clusters of gracefully drooping bell-shaped blossoms that are cream with a maroon streak down each petal. The flowers sit atop tall green stems having unusual ribbed, slightly twisting blue-gray leaves at the base. *A. siculum*, said to be deer resistant, is used as a seasoning in Bulgaria. The penetrating, skunky odor released when the plant is cut is attributed to the presence of butyl thiosulfinates, thought to originate from *S*-*n*-butylcysteine *S*-oxide (butiin) (*7*). Thiosulfinates containing methyl and 1-propenyl groups were also reported to be present (*7*).

The chemistry occurring when onions are cut is summarized in **Figure 2** (*1*, *2*). *S*-Alkenylcysteine *S*-oxide precursor **1a** (isoalliin) is cleaved by alliinase, splitting off aminoacrylic acid, which rapidly hydrolyzes to ammonium pyruvate. 1-Propenesulfenic acid (**2a**) is transformed by lachrymatory factor synthase (LFS) (*10*) to (*Z*)-propanethial *S*-oxide (**3a**), the onion LF. Sulfenic acid **2a**, **12a**, or **19** can also condense, giving thiosulfinates **4a**−**7a**, **9a**, **10a**, **13a**, and **20** or, in the case of self-reaction of **2a**, giving zwiebelanes, for example, **8a**. Bis-thial *S*-oxide **17** can also be formed from **2a** and **3a** (*1*, *2*), whereas cyclization of **1a** affords cycloalliin (**14a**). In all of these cases R = Me. Onion organosulfur compounds have two, three, four, or six carbon atoms reflecting various combinations of compounds with one and three carbon atoms.

The onion chemistry summarized in **Figure 2** took many years to be understood (*1*, *2*). It is relevant to genetically engineered "tearless onions," in which the LFS gene is silenced, leading to enhanced formation of compounds derived from **2a**, for example, **4a**−**10a** and **13a** (*11*), and the long-known (*12*), but little understood, phenomenon of "pinking of onions," now thought to involve compounds **4a**, **6a**, **9a**, **10a**, and **17** (*13*−*15*). Many of the onion compounds shown are thermally and hydrolytically unstable, leading to problems of artifact formation, for example, through the use of GC-MS with hot injection ports and/or the loss of material through reactions with solvents (*16*).

It is remarkable that two different enzyme systems, alliinase and LF synthase, are required to produce a unique C═S═O bonded natural product (a thial *S*-oxide or sulfine (*17*)), as represented by the onion LF. The electrophilic character of the LF together with its ability to react with thiols makes it an excellent candidate to activate TRPA1 (*6*) and TRPV1 (*5*), explaining its powerful irritant properties. Two other sulfines have thus far been reported to occur naturally, namely, compound **17**, from onion extracts as noted above, and phenylmethanethial *S*-oxide (**23**; **Figure 3**), from extracts of *P. alliacea*, a tropical weed extensively used in traditional medicine (*18*). Sulfine **23** is presumed to arise from action of a LF synthase on phenylmethanesulfenic acid **22** (*19*), in turn formed via alliinase cleavage of precursor **21** (*20*). Alternatively, **22** can self-condense, giving thiosulfinate **24** (*21*).

DART, one of several popular methods used for ambient ionization mass spectrometry, has attracted attention because of its ability to directly analyze gases, liquids, and solids in open air, without prior treatment (*22*, *23*). The DART source is generally used with a high-resolution time-of-flight mass spectrometer. DART is a "soft ionization" method, which for most compounds gives simple mass spectra that are easily obtained by momentarily holding the sample in the gas stream, either manually or using an automatic sampling device. Ionization under positive ion (PI-DART) conditions gives species formed when analytes collide

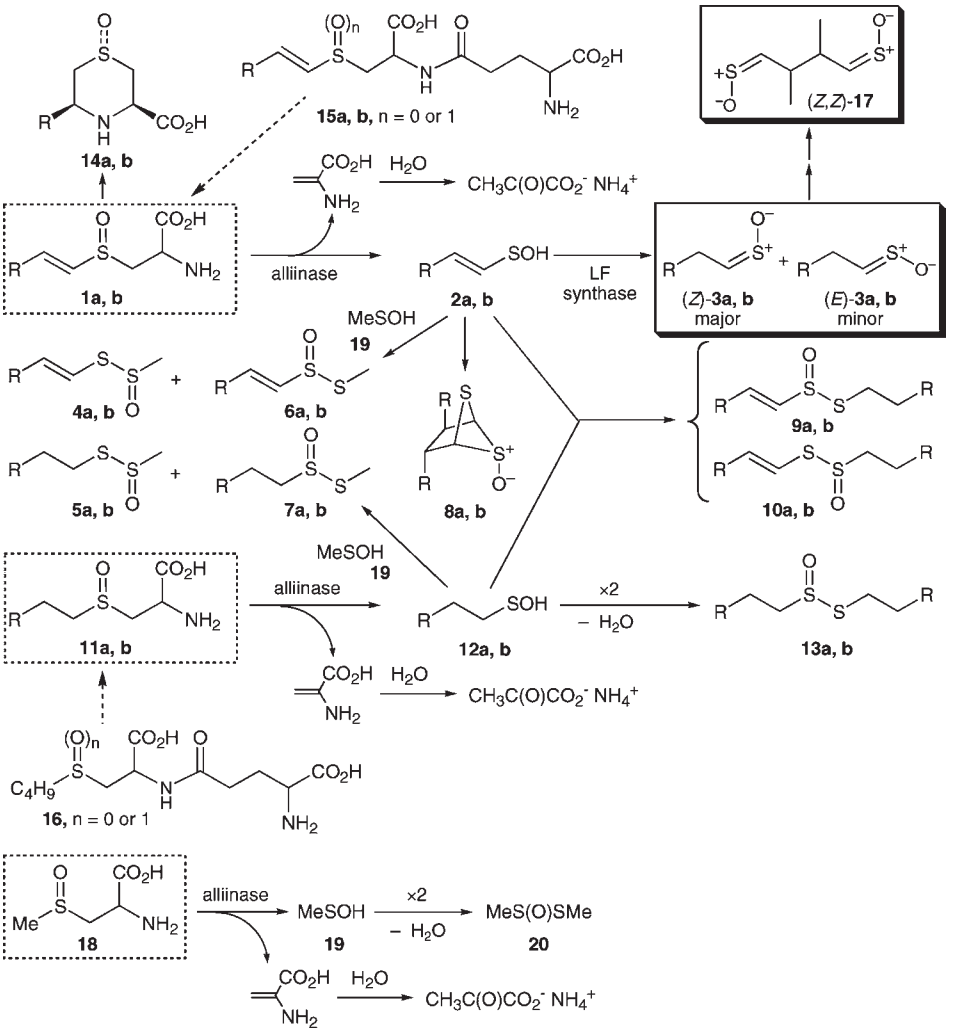

Figure 2. Comparison of proposed transformations for *A. cepa* (R = Me for **1a**−**15a**) and *A. siculum* (R = Et for **1b**−**15b**).

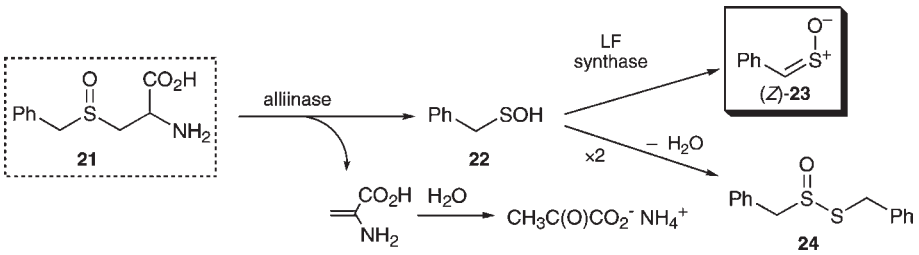

Figure 3. Proposed transformations for *Petiveria alliacea* (compounds **20**−**24**).

with protonated water clusters $[(H_2O)_n + H]^+$, producing an $[M + H]^+$ ion for analytes that have high proton affinities (*22*). The ability to perform high-resolution DART-MS under PI conditions without the need for sample preparation or solvent presents unique opportunities in food and natural products chemistry (*23*), allowing the direct observation of the rapid, complex cascade of enzymatically induced flavor-releasing processes that follow the wounding of plant cells. For example, capsaicin was easily measured as its $[M + H]^+$ species simply by slicing open a red pepper pod and passing the different parts of the pod in front of the ionizing beam (*22*). Other recent applications of DART-MS include identification of volatiles from eucalyptus (*24*), tumeric (*25*), dietary supplements (*26*), and an awake fly (*27*). We illustrate the utility of DART-MS in characterizing the reactive organosulfur compounds formed upon cutting onion and the woody and ornamental plants *P. alliacea* and *A. siculum*, respectively.

MATERIALS AND METHODS

General Methods. ^1H and ^{13}C NMR spectra were obtained using either a Bruker 400 MHz (for **3b**) or a Varian 500 MHz spectrometer [for **1b**, **14b**, **16** (n = 0)]. The chemical shifts are given in parts per million (ppm) relative to TMS at δ 0.00 ppm or $CDCl_3$ at δ 7.27 for proton spectra and relative to $CDCl_3$ at δ 77.23 for carbon spectra. GC-MS of **3b** was determined on a Varian Saturn 2100T (EI, 70 eV), using a 30 m × 0.25 mm i.d. × 0.25 μm VF-5 5% phenyl 95% dimethylpolysiloxane capillary column with 99.999% He as a carrier gas; initial column temperature = 60 °C, programmed after a 15 min hold to 260 °C; and injector temperature = 150 °C. IR spectra were determined using a Nicolet Impact 400 FTIR spectrometer (Thermo Fisher, Madison, WI); CD spectra were determined on a Jasco J-715 circular dichroism spectrometer (Jasco,

Tokyo, Japan). Specific rotation values were determined with an Optech PL1 polarimeter (Munich, Germany). 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), 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.

An AccuTOF-DART (JEOL USA, Inc., Peabody, MA) time-of-flight mass spectrometer (TOF-MS) was used for mass measurements. The mass spectrometer resolving power was 6000 (fwhm definition) measured for protonated reserpine. A mass spectrum of poly(ethylene glycol) with average molecular weight 600 was included in each data set as a reference standard for exact mass measurements. The atmospheric pressure interface was typically operated at the following potentials: orifice 1 = 20 V, orifice 2 = 3 V, ring lens = 3 V. In some experiments the voltage was reduced for orifice 1 to 12 V to minimize ion fragmentation. At these lower voltages, ion cluster formation was enhanced. The RF ion guide voltage was generally set to 550 V to allow detection of ions greater than m/z 55; in some experiments the voltage was adjusted to permit detection of ions as low as m/z 43. The DART ion source (IonSense Inc., Saugus, MA) was operated with room temperature helium gas at a flow rate of 2 L min^{-1}. The glow discharge needle was operated at 3500 V, the intermediate electrode (E1) at ±150 V, and the grid electrode at ±250 V. TSSPro3 software (Shrader Analytical, Detroit, MI) together with Mass Spec Tools programs (ChemSW Inc., Fairfield, CA) was used for data processing and data interpretation. In most experiments, an autosampler was used to precisely position a sample tube in the source region. Inverted melting point capillaries, which serve as sample tubes, were flamed before use to remove traces of lactic acid, found on skin, and other organic materials. With the inverted capillary positioned above the source area, the cleaned plant sample was repeatedly punctured by the capillary, disrupting the plant tissue. The capillary was then automatically lowered into the proper source position. With practice, DART measurements began within 1−2 s of sampling.

An Agilent dual ESI source ESI-MSD-TOF mass spectrometer at the Scripps Institute (La Jolla, CA) was used for accurate mass determination for compounds **1b**, **14b**, and **16**. A mixture of standards (Agilent ESI-TOF TUNE mix) was used to spray two lock masses at 121 and 922 from the second sprayer for internal calibration of each mass spectrum to get the highest mass accuracy. The sample was introduced by flow injection analysis at 4000 V using an 8 μL sample injection at 100 μL/min using an Agilent 1100 HPLC system with a solvent consisting of 50% methanol.

Plant Samples and Chemical Standards. Bulbs of the ornamental plant, *A. siculum*, were obtained from an Albany, NY, nursery or from Eurobulb (Zwanenburg, The Netherlands) in the summers of 2008 and 2009. The bulbs of *A. tripedale* were obtained from Dr. Leonid Bondarenko (Lithuanian Rare Bulb Garden, Vilnius, Lithuania) in September 2008. Voucher specimens are still cultivated in the Alliaceae species collection at University of South Bohemia and can be accessed upon request. The source of *P. alliacea* and the deposition of voucher samples are described elsewhere (*20*, *21*), as is the preparation of standard compounds **20** and **24** (*1*, *21*).

Extraction and Isolation of Natural Compounds. Fresh bulbs of *A. siculum* (827 g) were carefully peeled and homogenized in 1500 mL of MeOH/H_2O/HCl (90:9:1, v/v/v). 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 portion of 1500 mL of MeOH/H_2O/HCl (90:9:1, v/v/v), and the extracts were combined and concentrated to approximately 750 mL by vacuum evaporation (< 40 °C). After the pH had been adjusted to 2.5 by 10% NaOH, the precipitate formed was removed by centrifugation. The extract obtained was applied onto a cation-exchange column (25 × 5 cm; Amberlite IR-120, H^+ form, 16−45 mesh). After the column had been washed with 1% HCl (200 mL) and H_2O (500 mL), the amino acid-containing fraction was eluted with 0.1 M NaOH. Ninhydrin-positive fractions containing 1-BeCSO (checked by silica gel TLC, R_f 0.35 in 1-butanol/H_2O/CH_3COOH 12:5:3, v/v/v) were collected and freeze-dried. The residue obtained was redissolved in 150 mL of water, the pH was adjusted to 5.0 by 10% NaOH, and the solution was passed through an anion-exchange column (15 × 3 cm; Dowex 1X8, acetate form, 200−400 mesh). The target amino acid was not retained by the column. The eluent was freeze-dried, redissolved in 25 mL of 100 mM KH_2PO_4 buffer (pH 5.4), and subjected to preparative C-8 HPLC, with 10 mM KH_2PO_4 (pH 5.4, solvent A) and acetonitrile (solvent B) as the mobile phase. The gradient was as follows: A/B 97:3 (0 min), 97:3 (in 10 min), 65:35 (in 13 min), 65:35 (in 15 min), and 97:3 (in 20 min), with a flow rate of 18 mL min^{-1}. The fractions eluting at 8.0 min were collected, pooled, and freeze-dried, and the residue was carefully recrystallized from aqueous acetone to yield 312 mg of homoisoalliin (**1b**). Homocycloalliin (**14b**) was isolated in a manner similar to that for **1b** except that the anion-exchange chromatography step was skipped. **14b** eluted at 4.7 min under HPLC conditions identical to those used for the isolation of **1b**; 28 mg of **14b** was obtained from 376 g of the *A. siculum* bulbs.

(*E*)-/(*Z*)-Butanethial *S*-Oxide (3b). Treatment of *n*-butanesulfinyl chloride with triethylamine in ether at −78 °C followed by filtration and concentration as described for **3a** (*29*, *30*) afforded **3b**. GC-MS of the product (RT 5.73 min; 60 °C) showed m/z 104 (M^+; 27%) and 55 (100%); CH=SO group: (*Z*)-isomer ^{1}H NMR ($CDCl_3$) δ 8.17 (J = 8.0 Hz; 93.6%), (*E*)-isomer δ 8.74 (J = 9.3 Hz; 6.4%); ^{13}C NMR ($CDCl_3$) δ 177.89 (major), 170.07 (minor) (see Supporting Information). *E*/*Z* stereochemistry is assigned on the basis of previous work (*29*, *30*)). An earlier report (*28*) of the synthesis of **3b** (with MS and ^{1}H NMR data identical to those of (*Z*)-**3b**) did not define the stereochemistry or indicate the presence of a minor isomer (*E*)-**3b**.

A whole *A. siculum* plant (58 g) was homogenized with twice its weight of water using a Brinkmann Polytron PT3000, with icing to prevent overheating. The homogenate was vigorously shaken with ether (150 mL), layers were separated by centrifugation in sealed centrifuge tubes, and the intensely green ether layer was separated, dried ($MgSO_4$), and concentrated in an ice bath with a stream of dry argon to 1 mL and analyzed by GC-MS, NMR, and HPLC. The GC-MS was identical to that for synthetic **3b**; ^{1}H NMR ($CDCl_3$) showed δ 8.16 (CH=SO; J = 8.0 Hz; 93.6%) and (*E*)-isomer δ 8.82 (CH=SO; J = 9.3 Hz; 6.4%) (see Supporting Information). ^{1}H NMR multiplets in the δ 5.9−6.6 region indicated the presence of compounds containing C=C unsaturation.

Synthesis of Other Reference Compounds. *S*-Alk(en)yl-L-cysteine *S*-oxides, γ-glutamyl dipeptides, and their *S*-oxides were synthesized as described (*31*). The (S_S,R_{C3},S_{C5})-diastereomer of 3-carboxy-5-ethyl-1,4-thiazane *S*-oxide (homocycloalliin, **14b**) was prepared according to the method of Sakai and Yoneda (*32*). Isolation of a crude alliinase/LF synthase complex from the bulbs of *A. siculum* followed the procedure of Thomas and Parkin (*33*).

Analytical Data of the Identified Compounds. (*R_S,R_C,E*)-*S*-(*1-Butenyl)cysteine S-oxide* (***1b**; homoisoalliin, 1-BeCSO*): colorless solid; mp 155−158 °C; $[\alpha]^{22}_D$ +63° (*c* 0.36, H_2O); CD $\Delta\varepsilon_{max}$ (*c* 0.001, 22 °C, H_2O) + 4.79 (238 nm); ^{1}H NMR (D_2O) δ 0.95 (t, J = 7.5 Hz, 3H, H-8), 2.19 (m, 2H, H-7), 3.14 (dd, J = 8.0 and 14.0 Hz, 1H, H-3a), 3.33 (dd, J = 5.5 and 14.0 Hz, 1H, H-3b), 3.97 (dd, J = 5.5 and 8.0 Hz, 1H, H-2), 6.40 (dt, J = 1.7 and 15.4 Hz, 1H, H-5), 6.67 (dt, J = 6.2 and 15.4 Hz, 1H, H-6); ^{13}C NMR (D_2O) δ 11.3 (C-8), 25.3 (C-7), 50.8 (C-2), 53.0 (C-3), 128.8 (C-5), 149.6 (C-6), 172.0 (C-1); IR (KBr) 2913−2805, 1596, 1425, 1395, 1036, 964 cm^{-1}; ESI-TOF HRMS calcd for $C_7H_{13}NO_3S$ 192.0689 (M + H), found 192.0690. Assignment of the NMR spectra was aided by gCOSY and HETCOR measurements (see Supporting Information).

(*S_S,R_{C3},S_{C5})-3-Carboxy-5-ethyl-1,4-thiazane S-oxide* (***14b**; homocycloalliin*): colorless solid; mp 243−246 °C (hydrochloride); $[\alpha]^{22}_D$ −21° (*c* 0.55, H_2O); CD $\Delta\varepsilon_{max}$ (*c* 0.005, 22 °C, H_2O) + 0.24 (230 nm); ^{1}H NMR (D_2O) δ 0.91 (t, J = 7.6 Hz, 3H, H-9), 1.70 (m, 1H, H-8a), 1.82 (m, 1H, H-8b), 2.73 (dd, J = 12.5 and 15.5 Hz, 1H, H-6a), 2.94 (ddd, J = 0.7, 13.1, and 15.3 Hz, 1H, H-2a), 3.31 (dt, J = 2.8 and 15.5 Hz, 1H, H-6b), 3.51 (dt, J = 2.8 and 15.5 Hz, 1H, H-2b), 3.74 (m, 1H, H-5), 4.22 (dd, J = 2.8 and 13.1 Hz, 1H, H-3); ^{13}C NMR (D_2O) δ 8.6 (C-9), 25.5 (C-8), 43.7 (C-2), 44.1 (C-6), 48.0 (C-5), 49.5 (C-3), 171.3 (C-7); IR (KBr) 3080−2878, 1625, 1388, 1366, 1036 cm^{-1}; ESI-TOF HRMS calcd for $C_7H_{13}NO_3S$ 192.0689 (M + H), found 192.0693. Assignment of the NMR spectra was assisted by HETCOR and gCOSY measurements (see Supporting Information).

(*S_{C2},R_{C7}*)-*γ-Glutamyl-S-butylcysteine* (***16**)*: colorless hygroscopic solid; ^{1}H NMR (D_2O) δ 0.75 (t, J = 7.4 Hz, 3H, H-14), 1.24 (tq, J = 7.4 and 7.5 Hz, 2H, H-13), 1.42 (m, 2H, H-12), 2.06 (m, 2H, H-3), 2.42 (td, J = 7.8 and 2.6 Hz, 2H, H-4), 2.47 (t, J = 7.2 Hz, 2H, H-11), 2.79 (dd, J = 14.1 and 8.4 Hz, 1H, H-9a), 2.97 (dd, J = 14.1 and 4.7 Hz, 1H, H-9a), 3.81 (t,

Table 1. White Onion (*A. cepa*) PI-DART Measurements

species	neutral parent	measured	calcd	diff	abundance
$[C_3H_6SO + H]^+$	**3a** (LF)	91.0230	91.0218	0.0012	100
$[C_6H_{10}S_2O + H]^+$	**8a** (zwiebelanes)	163.0320	163.0251	0.0069	0.3
$[C_6H_{12}S_2O + H]^+$	PrSS(O)CH═CHMe (**9a**) PrS(O)SCH═CHMe (**10a**)	165.0403	165.0408	−0.0005	1.0
$[C_6H_{14}S_2O + H]^+$	**13a** PrS(O)SPr	167.0580	167.0564	0.0016	1.0
$[C_6H_{10}S_2O_2 + H]^+$	**17** bis-sulfine	179.0265	179.0201	0.0064	0.06

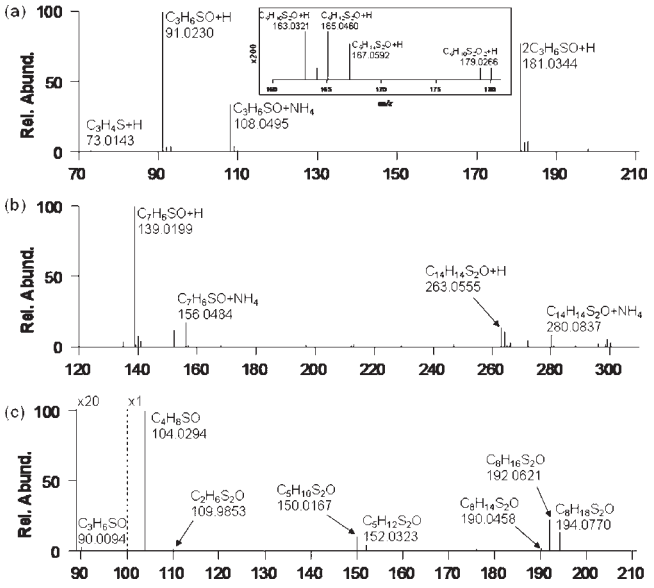

Figure 4. PI-DART traces from cut *A. cepa* (**a**), *P. alliacea* (**b**), and *A. siculum* (**c**) (for (**c**), simplified summed trace combining H^+ and NH_4^+ adducts shown as neutral parents).

J = 6.4 Hz, 1H, H-2), 4.45 (dd, J = 8.4 and 4.7 Hz, 1H, H-7); ^{13}C NMR (D_2O) δ 13.0 (C-14), 21.4 (C-13), 26.1 (C-3), 31.0 (C-12), 31.3 (C-4), 31.5 (C-11), 32.6 (C-9), 52.9 (C-7), 53.4 (C-2), 172.9 (C-5), 174.5 and 174.6 (C-1 and C-8); IR (KBr) 2959−2871, 1729, 1644, 1545, 1226, 960 cm^{-1}; ESI-TOF HRMS calcd for $C_{12}H_{22}N_2O_5S$ 307.1322 (M + H), found 307.1321. Assignment of the NMR spectra was assisted by HETCOR and gCOSY measurements (see Supporting Information).

GC-MS, HPLC, and LC-MS Analysis of *S*-Substituted Cysteine Derivatives. *S*-Substituted cysteines present in both *Nectaroscordum* species were analyzed by using the GC-MS method of Kubec and Dadáková (*31*), monitoring the following derivatives: *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 dansyl chloride derivatization (*31*). The identities of the γ-glutamyl dipeptides seen by HPLC were established by LC-MS (see Supporting Information).

RESULTS AND DISCUSSION

Allium cepa. The PI-DART mass spectrum for onion shows a predominant amount of LF **3a**, seen as a set of three intense ions at m/z 91 ($[C_3H_6SO + H]^+$), m/z 108 ($[C_3H_6SO + NH_4]^+$), and m/z 181 ($[(C_3H_6SO)_2 + H]^+$), as shown in **Figure 4a** and **Table 1**. In these spectra the LF reacts with a proton on a 1:1 or 2:1 basis or with ammonium ion, formed from the decomposition of aminoacrylic acid. Minor peaks are identified on the basis of their high -resolution (HR) masses and the following assumptions: (1) allyl compounds are present in onions at best at extremely low levels so that a single C_3H_5 group is most likely (*E*)-1-propenyl; (2) on the basis of prior studies of onion preparations (*1*, *2*), compounds of formula $C_6H_{10}S_2O$ and $C_6H_{10}S_2O_2$ are most likely zwiebelanes (**8a**) and bis-sulfine (**17**), respectively. On this basis, onions show as minor components ~0.2% zwiebelanes (**8a**), ~1% of mixed 1-propenyl propyl thiosulfinates (**9a**, **10a**) ($[(C_6H_{12}S_2O + H]^+$), ~1% of propyl propanethiosulfinate (**13a**, PrS(O)SPr; $[C_6H_{14}S_2O + H]^+$), and lower levels of bis-sulfine (**17**, [OSCHCHMeCHMeCHSO]; $[C_6H_{10}S_2O_2 + H]^+$). For **3a**, **8a**−**10a**, and **15a**, R = Me, with all formulas confirmed by HR-MS. Some onion samples also show traces of methyl/1-propenyl thiosulfinate regio- and stereoisomers (**4a**, **6a**; m/z 137, $[C_4H_8S_2O + H]^+$).

Table 2. *Petiveria alliacea* PI-DART Measurements

species	neutral parent	measured	calcd	diff	abundance
$[C_7H_6SO + H]^+$	**23**	139.0199	139.0218	−0.0019	17.3
$[C_7H_6SO + NH_4]^+$	**23**	156.0484	156.0483	0.0001	100
$[C_{14}H_{14}S_2O + H]^+$	**24**	263.0525	263.0564	−0.0039	17.6
$[C_{14}H_{14}S_2O + NH_4]^+$	**24**	280.0838	280.0830	0.0008	11.6
$[C_{14}H_{14}S_2 + H]^+$	$(BnS)_2$	247.0609	247.0615	−0.0006	2.8
$[C_{14}H_{14}S_2 + NH_4]^+$	$(BnS)_2$	264.0878	264.0881	−0.0003	13.6

Petiveria alliacea. The woody root of *P. alliacea* was abraded with a knife blade in the DART source region with a heated gas flow until a strong garlic-like odor was released from the plant. Under PI-DART conditions both **23** and **24** were seen as their protonated and ammoniated adducts (**Figure 4b** and **Table 2**). The presence of thiosulfinate **24** was confirmed with a synthetic standard (see Materials and Methods). A small peak corresponding to dibenzyl disulfide was also found. The higher temperatures required to volatilize **24** are presumably responsible for the formation of dibenzyl disulfide because di- and polysulfides were not seen when *Allium* samples were examined by DART at room temperature.

***A. siculum* Volatiles.** Samples examined using PI-DART showed the presence of (*E*)-/(*Z*)-butanethial *S*-oxide (**3b**) as the major component (**Figure 4c**; **Table 3**). The identity of **3b**, only the fourth sulfine known to occur naturally, was confirmed by synthesis. By NMR methods **3b** was found to be a mixture of 94% (*Z*)-**3b** and 6% (*E*)-**3b**. The synthesis of **3b** was previously reported, but only a single isomer of unknown stereochemistry was described (*28*). PI-DART also revealed the presence of moderate levels of 1-butenyl/methyl (**4b**−**7b**), 1-butenyl/butyl and dibutyl thiosulfinates (**4b**−**7b, 9b**/**10b**, and **13b**, respectively), and the higher homologue of zwiebelanes, **8b**, along with traces of onion LF **3a** and dimethyl thiosulfinate (**20**). For **3b**−**10b** and **13b** from *A. siculum*, R = Et. All formulas for the volatiles, which have two, four, five, or eight carbon atoms, reflecting various combinations of compounds with one or four carbon atoms, were confirmed by HR-MS. Significant quantities of disulfides or polysulfides were not detected by PI-DART and are therefore assumed not to be primary products. Compounds **5b**, **7b**, and **13b** have previously been isolated from *A. siculum* and fully characterized (*7*). Because compounds **3b**, **4b**, **6b**, and **8b**−**10b** have not been previously reported in nature, it would be desireable in future studies to confirm their formation in *A. siculum* by

Table 3. *Allium siculum* PI-DART Measurements

species	neutral parent	measured	calcd	diff	abundance
$[(C_3H_4O_3)_2 + NH_4]^+$	pyruvate	194.0707	194.0665	0.0042	3.3
$[C_3H_6SO + NH_4]^+$	**3a**	108.0438	108.0483	−0.0045	0.3
$[C_4H_8SO + H]^+$	**3b**	105.0378	105.0374	0.0004	78.6
$[(C_4H_8SO)_2 + H]^+$	**3b**	209.0659	209.0670	−0.0011	10.7
$[C_4H_8SO + NH_4]^+$	**3b**	122.0636	122.0640	−0.0004	100.0
$[C_2H_6S_2O + H]^+$	**20**	110.9932	110.9938	−0.0006	2.6
$[C_2H_6S_2O + NH_4]^+$	**20**	128.0197	128.0204	−0.0007	0.8
$[C_5H_{10}S_2O + H]^+$	**4b/6b**	151.0260	151.0251	0.0009	16.5
$[C_5H_{10}S_2O + NH_4]^+$	**4b/6b**	168.0497	168.0517	−0.0020	4.4
$[C_5H_{12}S_2O + H]^+$	**5b/7b**	153.0393	153.0408	−0.0015	7.6
$[C_5H_{12}S_2O + NH_4]^+$	**5b/7b**	170.0675	170.0673	0.0002	2.2
$[C_8H_{14}S_2O + H]^+$	**8b**	191.0534	191.0564	−0.0030	3.6
$[C_8H_{14}S_2O + NH_4]^+$	**8b**	208.0805	208.0830	−0.0025	1.0
$[C_8H_{16}S_2O + H]^+$	**9b/10b**	193.0697	193.0721	−0.0024	34.6
$[(C_8H_{16}S_2O)_2 + H]^+$	**9b/10b**	385.1351	385.1363	−0.0012	1.3
$[C_8H_{16}S_2O + NH_4]^+$	**9b/10b**	210.0953	210.0986	−0.0033	7.8
$[C_8H_{18}S_2O + H]^+$	**13b**	195.0850	195.0877	−0.0027	22.1
$[C_8H_{18}S_2O + NH_4]^+$	**13b**	212.1113	212.1143	−0.0030	4.5

synthesis and to determine their relative ratios, for example, by HPLC.

***A. siculum* and *A. tripedale* Nonvolatile Precursor Compounds.** Because isolation and separation of the trace quantities of new compounds **4b**−**10b** were not possible, efforts were made to isolate and characterize the likely precursors of these compounds, containing 1-butenyl and *n*-butyl groups. Amino acid-containing fractions from the bulbs of *A. siculum* and *A. tripedale* were obtained by extraction with aqueous methanol. Silica gel TLC of the isolated fraction from *A. siculum* revealed the abundant presence of a UV-absorbing, ninhydrin-positive compound, moving slightly slower than *S*-butylcysteine *S*-oxide (**11b**; butiin, BCSO). This compound was subsequently isolated by cation- and anion-exchange chromatography together with preparative C-8 HPLC.

^{13}C NMR spectroscopic data for the isolated compound indicated the presence of eight magnetically different carbons, with two of them being sp^2 hybridized (δ 128.8 and 149.6) and one being quarternary (δ 172.0). ^{1}H and ^{1}H−^{13}C NMR data showed the presence of the 1-butenyl group ($CH_3CH_2CH{=}CH-$) and the $-CH_2CH-$ moiety, containing two heterosteric methylene protons. Furthermore, ^{1}H NMR in DMSO-d_6 confirmed the presence of two groups of exchangeable protons (−COOH and $-NH_2$). The ^{1}H and ^{13}C NMR spectroscopic data (assisted by gCOSY and HETCOR measurements; see Supporting Information), together with a very strong IR absorption band at 1036 cm^{-1} (−S═O) and ESI-TOF-HRMS confirmed the structure of the isolated amino acid as *S*-(1-butenyl)cysteine *S*-oxide (**1b**; 1-BeCSO). The geometric configuration of the double bond was determined by ^{1}H NMR as (*E*), on the basis of the large coupling constant (J = 15.4 Hz) of the two CH═CH protons. CD and ^{1}H NMR spectroscopy were used to determine the absolute configuration at the two chiral centers (at the sulfur and the α-carbon). The CD spectrum of the amino acid showed a positive maximum ($\Delta\varepsilon_{max}$ +4.79, 238 nm); a Cotton effect of the same sign was also observed for the homologous (R_S,R_C,*E*)-*S*-(1-propenyl)cysteine *S*-oxide (isoalliin, **1a**, $\Delta\varepsilon_{max}$ +4.72, 237 nm) isolated from onion. The ^{1}H NMR spectrum 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} = 8.0 Hz and J_{BX} = 5.5 Hz) centered at δ 3.14 and 3.33, respectively. These values indicated that both the amino group and the sulfoxide oxygen are on the same face of the molecule (*7*). The structure of the isolated amino acid could thus be unambiguously determined as (R_S,R_C,*E*)-*S*-(1-butenyl)cysteine *S*-oxide (homoisoalliin, **1b**). Synthesis of **1b** has been reported by Müller and Virtanen (*8*) and later by Carson and co-workers (*34*).

Table 4. Content and Relative Ratios of *S*-Substituted Cysteine *S*-Oxides **18**, **1b**, and **11b** in Bulbs of the Subgenus *Nectaroscordum* Species, *A. siculum* and *A. tripedale*

	relative proportions (%) and total content (mg g^{-1} of fresh weight)			
species	MCSO (**18**)	1-BeCSO (**1b**)	BCSO (**11b**)	total content
A. siculum	32	65	5	5.3 ± 0.4
A. tripedale	23	13	64	8.9 ± 0.3

GC-MS analysis of the amino acid fractions of *A. siculum* and *A. tripedale* (fractions of the latter prepared as for *A. siculum*) also revealed the presence of other *S*-substituted cysteine derivatives, namely, the previously reported (S_S, R_C)-*S*-methylcysteine *S*-oxide (**18**; methiin, MCSO) and **11b** (butiin, BCSO). Interestingly, an isomer of **1b**, (*E*)-*S*-(2-butenyl)cysteine *S*-oxide (2-BeCSO), was also detected at trace levels (< 0.01 mg g^{-1} of fresh weight) in *A. siculum*. However, the occurrence of 2-BeCSO may be related to the extensive thermally induced (*E*/*Z*) isomerization of **1b**, which took place during the GC analysis. Contrary to the previous report of the presence of significant amounts of isoalliin (**1a**) in *A. siculum* (*7*), this compound could not be detected. However, our PI-DART detection of trace amounts of propanethial *S*-oxide (**3a**) requires the presence of trace amounts of **1a**. Identification of larger amounts of **1a** in *A. siculum* in the previous paper (*7*) was most likely erroneous.

The total content and relative proportions of the three major *S*-substituted cysteine *S*-oxides (MCSO, **18**; 1-BeCSO, **1b**; BCSO, **11b**) were determined in the bulbs of both members of the *Nectaroscordum* subgenus, namely, *A. siculum* and *A. tripedale* (**Table 4**). Although all three derivatives were present in both species at relatively high concentrations, their relative proportions differed significantly. Homoisoalliin (**1b**) was the predominant *S*-substituted cysteine *S*-oxide in *A. siculum*, whereas butiin (BCSO) was the major derivative found in *A. tripedale*.

That homoisoalliin (**1b**) serves as the precursor of the tear-producing butanethial *S*-oxide (**3b**) was proved by mixing **1b** with a crude enzyme preparation from the bulbs of *A. siculum*. The production of the sulfine was observed both organoleptically and by GC-MS. Although the mechanism was not studied in detail, it can be assumed that concerted action of both a C−S lyase (alliinase) and an LF synthase (*10*, *19*) is required for the formation of **3b** from homoisoalliin, as is the case for the lachrymators from onion and *P. alliacea* (*18*). In 1969 Müller and Virtanen reported that synthetic **1b** is cleaved by an enzymatic preparation from onion, giving a compound of formula C_4H_8SO, which they identified as 1-butenesulfenic acid (**2b**) (*8*), but which is much more likely **3b**.

Isolation of **1b** prompted us to search for the presence of additional amino acid derivatives analogous to those occurring in other *Allium* species (*1*−*3*). It has been shown that (*E*)-*S*-(1-propenyl)cysteine *S*-oxide (isoalliin, **1a**) is accompanied by a cyclic secondary amino acid (S_S,R_{C3},S_{C5})-3-carboxy-5-methyl-1,4-thiazane *S*-oxide (cycloalliin, **14a**) in onion and garlic. This unusual secondary amino acid was originally considered to be an artifact formed by cyclization of isoalliin under alkaline conditions during isolation. However, it was recently shown to be a component naturally present in *Allium* species, although its biochemical role is unknown (*35*). It could be assumed that an analogous compound might be possibly present also in *Nectaroscordum* species. Therefore, we prepared the next higher homologue of cycloalliin, (S_S,R_{C3},S_{C5})-3-carboxy-5-ethyl-1,4-thiazane

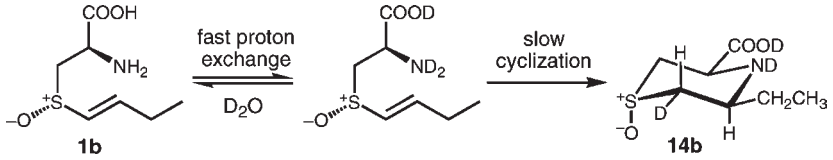

Figure 5. Deuterium exchange and cyclization of homoisoalliin **1b** to homocycloalliin **14b**.

S-oxide (homocycloalliin, **14b**), by stereospecific synthesis. Indeed, a compound having an HPLC retention time and UV spectrum identical to those of **14b** was present in the amino acid fraction obtained from the bulbs of *A. siculum*. This compound was subsequently isolated by ion-exchange chromatography and preparative C-8 HPLC. It exhibited identical spectral properties [^{1}H and ^{13}C NMR (including HETCOR and gCOSY 2D spectra), CD, and optical activity] with the sample of synthetic **14b**. Although this compound was also detected in an extract prepared under very mild extraction conditions (cold 90% methanol, no ion-exchange chromatography treatment), we cannot confirm its presence in the intact tissue with certainty because of the striking propensity of homoisoalliin (**1b**) to cyclize (*34*). For example, we observed that a pure sample of **1b** in D_2O was partially converted into deuterium-labeled **14b** during an overnight NMR experiment. In 3 weeks, the extent of conversion of **1b** into **14b** reached nearly 65% at 23 °C, with **14b** being the only decomposition product detectable by ^{1}H NMR. On the basis of the NMR data, the deuterium in **14b** was selectively incorporated in the equatorial position at C-6 (see Supporting Information) (**Figure 5**).

S-Substituted cysteine *S*-oxides are typically accompanied by the corresponding γ-glutamyl dipeptides in the plant tissue. These compounds are intermediates in the biosynthesis of *S*-alk(en)ylcysteine *S*-oxides, among other functions (*36*). Therefore, we searched for the presence of such dipeptides in *Nectaroscordum* species. Indeed, all six expected compounds, γ-glutamyl-(*E*)-*S*-(1-butenyl)cysteine and its *S*-oxide (**15b**, $n = 0$ and $n = 1$, respectively), γ-glutamyl-*S*-butylcysteine and its *S*-oxide (**16**, $n = 0$ and $n = 1$, respectively), and γ-glutamyl-*S*-methylcysteine and its *S*-oxide, were found in both *Nectaroscordum* species by LC-MS (see Supporting Information). Due to a limited amount of plant material, isolation of these dipeptides from the bulbs was not attempted. The identification of **15b** and **16** was tentatively made on the basis of LC-MS data and by comparison of retention times and UV spectra with those of authentic compounds prepared by synthesis (in the case of the *S*-methyl and *S*-butyl derivatives). Although the dipeptides were not isolated for detailed spectral analysis, it can be assumed that all of them are composed of L-amino acids and thus have the absolute configuration (S_{C2},R_{C7}) around the two chiral carbons. Compounds **1b**, **14b**, **15b**, and **16** have not been previously reported in nature.

Our work underscores the utility of DART-MS as a powerful tool in natural products chemistry. DART-MS was used to establish the formation when *A. siculum* is cut of a family of organosulfur compounds, previously unknown in nature, quite remarkably containing one more carbon than related compounds similarly formed from onions. Our findings also have significant implications for the taxonomy of the genus *Allium*. *S*-Substituted cysteine derivatives with a four-carbon side chain have not been reported to occur in any other *Allium* plant except for the two *Nectaroscordum* species (*37*, *38*). The occurrence of homoisoalliin (**1b**), butiin (**11b**), and the four corresponding γ-glutamyl dipeptides [**15b** ($n = 0$, 1), **16** ($n = 0$, 1)] thus seems to be a unique biochemical feature of only these two plants. It represents a significant additional argument in support of separating *Nectaroscordum* species at a generic level as an individual group.

ABBREVIATIONS USED

CD, circular dichroism; DART-MS, direct analysis in real time−mass spectrometry; ESI, electrospray ionization; gCOSY, gradient-selected correlation spectroscopy; HETCOR, heteronuclear chemical shift correlation; LC-MS, liquid chromatography−mass spectrometry; GC-MS, gas chromatography−mass spectrometry; HPLC, high-performance liquid chromatography; HR-MS, high-resolution mass spectrometry; LF, lachrymatory factor; LFS, lachrymatory factor synthase; PI, positive ionization; RT, retention time; TOF-MS, time-of-flight mass spectrometer; TRPA1, transient receptor potential ion-channel protein, member A1; TRPV1, transient receptor potential ion-channel protein, member V1.

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Supporting Information Available: ^{1}H NMR spectra of natural and synthetic **3b**; GC-MS of **3b**; ^{1}H and ^{13}C NMR and IR spectra of **1b**, **14b**, and **16**; CD of **1b** and **14b**; HETCOR of **14b** and **16**; gCOSY of **1b** and **14b**; and LC-MS spectra of **1b**, **14b**, **15b** ($n = 0$, 1), and **16** ($n = 0$, 1). This material is available free of charge via the Internet at http://pubs.acs.org.

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