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J. Nat. Prod., **1993**, 56 (6), 864-869• DOI:
10.1021/np50096a009 • Publication Date (Web): 01 July 2004

Journal of Natural Products
Vol. 56, No. 6, pp. 864-869, June 1993

A NOVEL AMINO ACID GLYCOSIDE AND THREE AMINO ACIDS FROM *ALLIUM SATIVUM*

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ABSTRACT.—A novel amino acid glycoside, (−)-*N*-(1′-deoxy-1′-β-D-fructopyranosyl)-*S*-allyl-L-cysteine sulfoxide [**1**], was isolated from a hydrophilic extract of the leaves of *Allium sativum* (Alliaceae), together with three known compounds: (+)-*S*-allyl-L-cysteine sulfoxide [**2**], (+)-*S*-methyl-L-cysteine sulfoxide [**3**], and (+)-*S*-(*trans*-1-propenyl)-L-cysteine sulfoxide [**4**]. The latter two substances were isolated for the first time from garlic. The structure elucidation of **1** was performed by extensive chemical, spectroscopic (ir, fabms, 1D and 2D ^{1}H- and ^{13}C-nmr measurements) and chromatographic experiments. For the first time, detailed nmr data and the unambiguous assignment of all ^{13}C-nmr data for compounds **2–4** were reported. Compound **1**, the amino acid glycoside, revealed a significant inhibition of in vitro platelet aggregation induced by ADP and epinephrine, whereas none of the amino acids tested displayed any activity.

Bulbs of *Allium sativum* L. (Alliaceae) are cultivated world-wide for medicinal purposes and as a spice. Their characteristic constituents are cysteine derivatives (1,2), whereby the corresponding γ-glutamyl dipeptides (3) and the cysteine sulfoxides attained particular significance. The latter act as substrates of the enzyme alliinase (4) and are therefore precursors of a cascade of pharmacologically active compounds formed after enzymatic conversion (5,6). The transformation products of (+)-*S*-allyl-L-cysteine sulfoxide, allicin, the ajoenes, and diallyl disulfide, showed significant inhibition of in vitro platelet aggregation (7,8), whereas from the genuine compounds, only alliin was tested until recently (9) and revealed only a slight activity (IC_{50} 60 μM) using ADP and collagen as inducers. Additionally a series of in vitro tests determined the antimicrobial activity of aqueous garlic extracts and pure transformation products (10–12). In phytotherapy, various clinical studies delineated the efficacy of standardized garlic powder in lowering plasma levels of triglycerides, cholesterol, and phospholipids and in decreasing spontaneous thrombocyte aggregation and plasma viscosity (13–15).

No phytochemical investigation was reported about the genuine constituents of the aerial part of garlic until recently. In this contribution a simple isolation procedure under gentle conditions using chromatographic methods was developed to obtain the genuine sulfur-containing amino acids detected in a polar leaf extract as pure compounds. Because most of the known substances have been isolated from *Allium* species in the 1960s or earlier (16–19), nmr data, expecially ^{13}C-nmr data for their structure elucidation, were lacking. We could assign all nmr values of the isolated compounds **1–4** by means of 1D and 2D nmr techniques. In addition we were able to get some new information about the pharmacological activity of compounds **1–4**.

RESULTS AND DISCUSSION

Inactivation of enzymes present in the plant tissues was performed with a high amount of MeOH (80%) in the extraction medium and by maceration at low temperature (2°), while more lipophilic compounds were removed by subsequent extraction with

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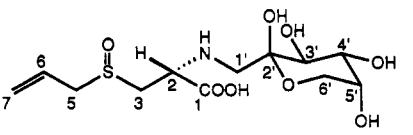

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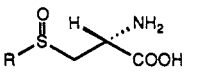

2 R=-CH_2CH=CH_2
3 R=Me
4 R=-CH=CHMe

CH_2Cl_2 and EtOAc. The remaining aqueous fraction was chromatographed over a strong acidic cation exchanger to concentrate the amino acids selectively. Further purification using gel permeation chromatography and subsequent chromatography on reversed-phase C-18 yielded predominantly compounds **1–4**.

The hygroscopic compound **1** was suggested to have the molecular formula $C_{12}H_{21}NO_8S$ from fabms. It gave a positive ninhydrin reaction (yellowish-purple color). Its determination by reversed-phase hplc after pre-column derivatization with 9-fluorenylmethyl chloroformate (20) indicated the presence of a secondary amine, while no reaction product could be obtained by using the reagent for derivatizing exclusively primary amines (20,21), *ortho*-phthaldialdehyde/*tert*-butanethiol. From the ir spectroscopic data the presence of a carboxyl group (1670 cm^{-1}) and of a sulfoxide moiety (1020 cm^{-1}) was implied, the first accounting for the amino acid part of the molecule. Acid hydrolysis of **1** and subsequent tlc analysis yielded alliin [**2**] and fructose along with three minor degradation products.

The ^{1}H-nmr spectrum of **1** indicated a discrete spin system of five protons comprising an allyl group. The high frequency shift of the two protons of the methylene group at δ 3.67 (br d, J=13.4 Hz) and δ 3.85 (dd, J=7.9, 13.4 Hz) located them at a strongly electron withdrawing structural element (sulfoxide group). The latter of them coupled to the proton at δ5.92 (m, J=6.9, 7.9, 10.2, 17.0 Hz), which in turn coupled to the protons of the C-7 methylene group (δ 5.48, br, d, J=17.0 Hz, and δ 5.55, br d, J=10.2 Hz). Other structural features obvious from both the ^{13}C- (Table 1) and ^{1}H-nmr data were the presence of a further methylene group [δ 3.45 and δ 3.31, 49.6 (t) ppm] linked to the sulfoxide group and to the α-carbon atom of the amino acid [60.1 (d) ppm]. From the resonances of the carbon signals, the remaining fragment of **1** was identified as a fructose moiety. From the coupling constants of H-3′, H-4′, and H_b-6′ (^3J=9.9 Hz, ^2J=12.2 Hz), it was assumed that the fructose part is present as a pyranoid ring system system (22). The ^{1}H-^{1}H COSY experiment allowed for the assignment of the coupling pattern of the protons. ^{1}H-^{13}C 2D inverse short range correlation experiments permitted the association of all protons with the carbon atoms to which they were directly attached. The long-range ^{1}H-^{13}C 2D correlation spectrum delineated connectivities between protons and carbons separated by two or three bonds. Thus, the linkage between the C-1′ of the sugar unit and the amino group of the cysteine sulfoxide derivative, and further the proposed connectivities of the sugar moiety, were proven, In addition we were able to assign all quaternary carbons. The relevant ^{1}H-^{13}C correlations that allowed the assignments of the quaternary carbon resonances for compound **1** are: H-2 (δ 4.42) is coupled to C-1 (171.7 ppm), H-3′ (δ 3.73) to C-2′ (96.9 ppm).

In aqueous solution compound **1** showed mutarotation due to the free hydroxyl

TABLE 1. ^{13}C nmr (75.5 MHz, D_2O) Data for Compounds **1–4**.[a]

Carbon	Compound			
	1	**2**	**3**	**4**
C-1	171.7 s	173.0 s	173.0 s	173.0 s
C-2	60.1 d	52.4 d	52.6 d	52.2 d
C-3	49.6 t	51.3 t	54.5 t	54.1 t
C-5	56.7 t	56.7 t	39.7 q	132.2 d
C-6	125.8 d	125.6 d		145.4 d
C-7	127.1 t	126.5 t		19.2 q
C-1′	54.2 t			
C-2′	96.9 s			
C-3′	71.5 d			
C-4′	70.9 d			
C-5′	70.4 d			
C-6′	65.6 t			

[a]Chemical shifts are reported in ppm with reference to *tert*-BuOH as internal standard (31.2 ppm); multiplicities by DEPT.

group at C-2′. In the ^{13}C-nmr spectrum the signals at higher frequencies of the resonances of the α- and β-D-fructofuranose forms (23) could be observed as minor signals.

The compound exhibited three chiral centers. The assignments of the two in the alliin part of the molecule were in agreement with both isolated and synthetic (*S*)-(+)-*S*-allyl-L_R-cysteine sulfoxide. The third center belongs to fructose. No information about its absolute configuration could therefore be deduced from the ^1H-nmr spectrum. From the molecular rotation difference (+27.1°) (24) and the chemical shift values of the carbon resonances the β-D configuration was assigned. Consequently, the structure of **1** was established as (−)-*N*-(1′-deoxy-1-β-D-fructopyranosyl)-*S*-allyl-L-cysteine sulfoxide, a novel, unusual amino acid glycoside.

Compound **2** was identified on the basis of its chromatographic behavior and its spectral data in comparison to synthetic (+)-*S*-allyl-L-cysteine sulfoxide. Furthermore the ^{13}C-nmr signals of **2** were unambiguously assigned for the first time. The essential problem was the assignment of the chemical resonances of the two triplets (C-3, C-5) both adjacent to the sulfoxide group. From a ^1H-^{13}C short range correlation experiment, their position could be deduced unambiguously: H_2-3 [δ 3.21 (dd) and δ 3.43 (dd)] correlate to C-3 (51.3 ppm) and H_2-5 [δ 3.63 (dd) and δ 3.84 (dd)] to C-5 (56.7 ppm). The chemical shift values of both carbons are deshielded when compared to the corresponding resonances of the carbon atoms linked to the reduced sulfur (thioether form) (3, 21). Additionally, the oxidation of the sulfur produced a second chiral center that was the predominant influence on the optical rotation. It is identical to those of alliin synthesized from L-cysteine without cleavage of the bonds at the chiral carbon C-2 and with data from the literature (19,25). Compound **2** was therefore identified as (+)-*S*-allyl-L-cysteine sulfoxide.

Compound **3** was identified as (+)-*S*-methyl-L-cysteine sulfoxide on the basis of spectroscopic evidence (fabms, ^1H and ^{13}C nmr) and in comparison to synthetic (±)-*S*-methyl-L-cysteine sulfoxide. The positive rotation angle of **3** suggested it to have the same configuration as reported for **2**.

For compound **4** the molecular formula, $C_6H_{11}NO_3S$, was compatible with data from fabms. The positive ninhydrin reaction (purple color) on tlc indicated the presence of an amino acid. The ir signal at 1000 cm^{-1} is characteristic for a sulfoxide group (26).

From the ^{1}H-nmr data of **4** two discrete spin systems could be discerned. Thus, the proton at δ 6.53 (dd, J=1.5, 15.2 Hz) coupled to the proton at δ 6.71 (br dd, J=6.7, 15.2 Hz), which in turn coupled to the protons of the C-7 methyl group (δ 1.94, dd, J=1.5, 6.7 Hz) giving rise to the C-5 to C-7 fragment, the propenyl group. The stereochemistry of the $\Delta^{5,6}$ double bond was *E* on the basis of the coupling constant (J=15.2 Hz). The resonances of the protons at δ 3.45 (dd, J=5.5, 14.0 Hz) and δ 3.25 (dd, J=8.0, 14.0 Hz) were located as part of an ABX system; they formed the second proton spin system and were assigned to the methylene group of cysteine. The proton connected to the α-carbon atom (X) caused a quartet at δ 4.11 (dd, J=5.5, 8.0 Hz). The absolute configuration at the chiral centers was determined to be similar to that of compound **2**. Compound **4** is an isomer of **2** with regard to the propenyl double bond and was characterized as (+)-*S*-(*trans*-1-propenyl)-L-cysteine sulfoxide.

The isolated genuine compounds were screened in various in vitro test systems. Antimicrobial activities were reported for the transformation products of the L-cysteine derivatives only (10–12). We tested compound **1** as well as the genuine prodrugs, compounds **2–4**, by tlc-bioautographic tests (27) using *Penicillium oxalicum* (CBS 219.30), *Bacillus subtilis* (ATCC 6633), *Micrococcus luteus* (ATCC 9341), and *Escherichia coli* (ATCC 25922) as test organisms. None of the test compounds showed antibacterial or antifungal activities at the concentration tested (25 μg).

Additionally the inhibition of thrombocyte aggregation was evaluated in vitro for a series of cysteine derivatives with ADP, collagen, and epinephrine as inducers (Table 2). Previous results reporting for alliin an IC_{50} value of 60 μM (9) could not be confirmed. Under the conditions tested, compound **1** was the only active substance. The thrombocyte aggregation triggered by ADP and epinephrine was significantly inhibited at an inhibitor concentration of 1 mM. While using collagen as an inducer, only a slight inhibition was observed, but the lag-time of the thrombocytes was increased up to 50–70% and their shape-change almost failed to appear. Although the inhibition effect of **1** is lower when compared to the alliin transformation products [e.g., ajoenes: IC_{50} 95 μM (8)], it could be stated that a genuine garlic constituent displayed some biological activity.

TABLE 2. Maximal Platelet Aggregation (%) of Compound **1**.[a]

Compound (1 mM)	Aggregation (%) ±SD		
	Epinephrine (2 μM)	ADP (2 μM)	Collagen (1 μg/ml)
1	40.2±14.1	39.2±21.8	82.0±3.4
Control	75.0±17.7	69.0±7.5	88.6±7.7

[a]Mean values of 4 determinations.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—^{1}H- and ^{13}C-nmr spectra were obtained at 300 MHz and 75.5 MHz (Bruker AMX-300), respectively, with *tert*-BuOH as internal standard (^{1}H-nmr δ 1.22; ^{13}C nmr 31.2 ppm) and D_2O as solvent. 2D heteronuclear short range correlated spectra were measured in the inverse mode (28) at 300/75.5 MHz with the relevant delays optimized for J_{CH} 136 Hz for compounds **1** and **2**. 2D homonuclear and heteronuclear correlated nmr experiments were performed at 600/150 MHz for **1** (Bruker AMX-600); the relevant delays of the inverse long-range correlated spectrum (29) were optimized for J_{CH} 10 Hz; fabms were measured on a ZAB2-SEQ instrument at 8.3 keV and with glycerin as a matrix; ir spectra were recorded in KBr on a Perkin-Elmer 781 infrared spectrometer; and melting points were measured with a Mettler FP 61 apparatus. The optical rotations were measured with a Perkin-Elmer 141 polarimeter using H_2O as solvent. For cc, Amberlite IR-120 (H^+), Sephadex LH-20, and RSil RP-18 HL (15–35 μm) were

used as stationary phases. Semi-preparative hplc was performed with a Waters 6000A solvent delivery system connected to a Rheodyne 7125 injector and a Perkin-Elmer LC-55 UV/VIS spectrometer (λ 210 nm or 220 nm). The column used was filled with Spherisorb ODS II, 5 μm (Knauer, 250×16 mm). The mobile phase for the semipreparative separations was optimized using analytical hplc [column: Spherisorb ODS II, 5 μm (Knauer, 250×4 mm), with a HP 1090M liquid chromatograph, and a HP 1040 diode array detector]. Aluminum plates coated with Si gel 60 F_{254} (0.2 mm thick, Merck) were used for tlc.

PLANT MATERIAL.—Garlic was cultivated in Lindau, Switzerland, and cloves were purchased at a local supplier. The garlic leaves were collected. A freeze-dried voucher specimen is deposited at the Department of Pharmacy, ETH-Zurich.

EXTRACTION AND PRE-PURIFICATION.—The freeze-dried, powdered leaves (3.2 kg) were macerated with MeOH 80% (4×2 days, each step with 12 liters at 2°). After concentration to about 4 liters, the aqueous phase was extracted with CH_2Cl_2 (3×4 liters) and EtOAc (3×4 liters). The remaining aqueous layer was freeze-dried and afforded a residue of 670.3 g. From the column (600 ×53 mm) filled with the strong acidic cation exchanger, Amberlite IR-120, the amino acids of 120 g of the H_2O extract were eluted with 0.75 M NH_4OH (9.9 g). By gel permeation chromatography on Sephadex LH-20 (800×33 mm, pre-column 100×10 mm) 2.1 g of the amino acid fraction was separated into two fractions by increasing the amount of MeOH in H_2O from 0% to 20% within 20 h. This purification step was repeated twice.

ISOLATION.—Separation of Sephadex fraction 1 (300 mg) by semi-preparative hplc (H_2O) yielded, after freeze-drying, 27 mg of **3** and 140 mg of **2** as pure compounds. Semi-preparative hplc (0.1% aqueous trifluoracetic acid) of Sephadex fraction 1 (540 mg) afforded after freeze-drying 86 mg of pure **1** and 33 mg of pure **4**. Lplc on RP-18 (800×26 mm; pre-column 100×10 mm; 0.1% aqueous trifluoracetic acid, 1 ml/min) of Sephadex fraction 1 (1 g) produced a main fraction that afforded, after freeze-drying and crystallization from Me_2CO/H_2O, 245 mg of **2**.

*(−)-*N*-(1′-Deoxy-1′-*β*-*D*-fructopyranosyl)-*S*-allyl-*L*-cysteine sulfoxide* [**1**].—Compound **1** (86 mg, white powder): $[\alpha]^{20}D$ −25.4° (*c*=0.410, H_2O); ir ν max (KBr) 3380, 1670, 1020, 930 cm^{-1}; fabms *m/z* $[2M+H]^+$ 679, $[M+H]^+$ 340; ^1H nmr (D_2O) δ 3.31 (m, 1H, H_b-3), 3.31–3.48 (m, 2H, H_2-1′), 3.45 (br d, 1H, *J*=6.7 Hz, H_a-3), 3.67 (br d, 1H, *J*=13.4 Hz, H_b-5), 3.73 (d, 1H, *J*=9.9 Hz, H-3′), 3.75 (br d, 1H, *J*=12.2 Hz, H_b-6′), 3.85 (dd, 1H, *J*=7.9, 13.4 Hz, H_a-5), 3.87 (br d, 1H, *J*=9.9 Hz, H-4′), 3.97 (br s, 1H, H-5′), 4.02 (m, 1H, H_a-6′), 4.42 (br t, 1H, *J*=6.7, 7.6, Hz, H-2), 5.48 (br d, 1H, *J*=17.0 Hz, H_{trans}-7), 5.55 (br d, 1H, *J*=10.2 Hz, H_{cis}-7), 5.92 (m, 1H, *J*=6.9, 7.9, 10.2, 17.0 Hz, H-6); ^{13}C nmr see Table 1.

Alliin [**2**].—Compound **2**, (+)-*S*-allyl-L-cysteine sulfoxide (245 mg, white needles from Me_2CO/H_2O): $[\alpha]^{20}D$ +60.8° (*c*=0.559, H_2O); ir ν max (KBr) 3400, 3000, 1590, 1020, 920 cm^{-1}; fabms *m/z* $[2M+H]^+$ 377, $[M+H]^+$ 178; ^1H nmr (D_2O) δ 3.21 (dd, 1H, *J*=7.6, 14.0 Hz, H_b-3), 3.43 (dd, 1H, *J*=6.2, 14.0 Hz, H_a-3), 3.63 (dd, 1H, *J*=7.9, 13.4 Hz, H_b-5), 3.84 (dd, 1H, *J*=7.0, 13.4 Hz, H_a-5), 4.20 (dd, 1H, *J*=6.2, 7.6 Hz, H-2), 5.48 (br d, 1H, *J*=17.1 Hz, H_{trans}-7), 5.53 (m, 1H, *J*=10.3 Hz, H_{cis}-7), 5.91 (m, 1H, *J*=7.0, 7.9, 10.3, 17.1 Hz, H-6); ^{13}C nmr see Table 1. *Anal.* calcd for $C_6H_{11}NO_3S \cdot {}^1/_2H_2O$: C 38.7, H 6.4, N 7.5. Found C 38.7, H 6.1, N 7.3. Mp 162° (dec.).

*(+)-*S*-Methyl-L-cysteine sulfoxide* [**3**].—Compound **3** (27 mg, white powder): $[\alpha]^{20}D$ +118.2° (*c*=0.322, H_2O); ir ν max (KBr) 3490, 3100, 1690, 1000 cm^{-1}; fabms *m/z* $[2M+H]^+$ 303, $[M+H]^+$ 152; ^1H nmr (D_2O) δ 2.80 (s, 3H, H_3-5), 3.22 (dd, 1H, *J*=7.8, 14.0, Hz, H_b-3), 3.47 (dd, 1H, *J*=6.0, 14.0 Hz, H_a-3), 4.19 (dd, 1H, *J*=6.0, 7.8 Hz, H-2); ^{13}C nmr see Table 1.

*(+)-*S*-(trans-1-Propenyl)-L-cysteine sulfoxide* [**4**].—Compound **4** (33 mg, white powder): $[\alpha]^{20}D$ +70.9° (*c*=0.308, H_2O); ir ν max (KBr) 3400, 2950, 1610, 1000, 945 cm^{-1}; fabms *m/z* $[M+Na]^+$ 200, $[M+H]^+$ 178; ^1H nmr (D_2O) δ 1.94 (dd, 3H, *J*=1.5, 6.7 Hz, H_3-7), 3.25 (dd, 1H, *J*=8.0, 14.0 Hz, H_b-3), 3.45 (dd, 1H, *J*=5.5, 14.0 Hz, H_a-3), 4.11 (dd, 1H, *J*=5.5, 8.0 Hz, H-2), 6.53 (dd, 1H, *J*=15.2, 1.5 Hz, H-5), 6.71 (br dd, 1H, *J*=6.7, 15.2 Hz, H-6); ^{13}C nmr see Table 1.

INHIBITION OF PLATELET AGGREGATION.—The test was performed according to the turbidimetric method (30) with human blood from 4 healthy volunteers who avoided any medical treatment for 14 days. A dual channel aggregometer (LAbor, Ahrensburg, F.R.G.) was calibrated with platelet-poor plasma (PPP; 100% transmission) and platelet-rich plasma (PRP; 0% transmission). The incubation started 3 min before the aggregation was induced by adding ADP, epinephrine, or collagen. The test solution had the following composition: 250,000 platelets/μl plasma, 1 mM of the test compound (0.9% aqueous NaCl as solvent), and 2 μM ADP, 2 μM epinephrine, or 1 μg/ml collagen. The increase of the light transmission was measured as a factor of time. Inhibition of the aggregation was calculated in comparison to the control samples. A significant difference between the test and control experiments was determined by means of the two-tailed Student's *t*-test (P=0.05).

ACKNOWLEDGMENTS

Financial support from Alpha foundation (Fribourg, Switzerland) is gratefully acknowledged. We thank Dr. S.J. Ziegler for the synthesis of alliin and (±)-*S*-methyl-L-cysteine sulfoxide. For the nmr measurements we thank Dr. D. Moskau (Spectrospin AG, Fällanden, CH) and Dr. A.D. Wright (Department of Pharmacy, ETH-Zürich, CH). Our thanks are also due to Mr. R. Häfliger (Department of Chemistry, ETH-Zürich, CH) for fabms measurements.

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Received 20 October 1992