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Antineoplastic Agents. 570. Isolation and Structure Elucidation of Bacillistatins 1 and 2 from a Marine *Bacillus silvestris*, †,‡

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Abstract

Two new cyclodepsipeptides designated bacillistatins 1 (**1**) and 2 (**2**) have been isolated from cultures of a sample of *Bacillus silvestris* that was obtained from a Pacific Ocean (southern Chile) crab. Each 12-unit cyclodepsipeptide strongly inhibited growth of a human cancer cell line panel, with GI₅₀s of 10^{−4}–10^{−5} µg/mL, and each compound was active against antibiotic-resistant *Streptococcus pneumoniae*. The structures were elucidated by a combination of X-ray diffraction and mass and 2D NMR spectroscopic analyses, together with chemical degradation.

Marine microorganisms are rapidly becoming a very useful source of new cancer cell growth inhibitory substances that have unique structures. Illustrative are recent examples of antineoplastic substances from marine bacteria,^{1a–h} fungi,^{2a–h} cyanobacteria,^{3a–e} and dinoflagellates.^{4a–d} As part of our extended evaluation of terrestrial and marine microorganisms as sources of new anticancer drug candidates, we collected a marine crab on Chiloé Island, Chile, in 1998. A *Bacillus* species, subsequently identified as *B. silvestris*, was isolated from the crab, and extraction of the scaled up bacterial broth has led to the identification of two new cyclodepsipeptides with antibacterial and human cancer cell line inhibitory activity. Bioactive cyclodepsipeptides have been isolated previously from other *Bacillus* species, including *B. cereus*,^{5a, d} *B. polymyxa*,^{5c} and *B. natto*.^{5b} Members of the genus *Bacillus* are common in both terrestrial and marine sediments. *Bacillus silvestris* was first described in 1999, when it was isolated from a sample of forest soil in Germany,^{6a} and more recently it was identified in water samples taken from the southern Baltic Sea, a brackish environment.^{6b}

Results and Discussion

The *Bacillus silvestris* culture was scaled up and extracted as described in the Experimental Section to give a dark-brown gum (3.14 g; P388 lymphocytic leukemia: ED₅₀ 0.0066 µg/mL), which was shown by HPLC analysis to comprise a mixture with at least six closely spaced peaks. Subsequent high-resolution LC-MS showed the mixture to be more complex than was apparent from the HPLC analysis (Table 1).

Attempts at separation by way of gel permeation and partition chromatography using Sephadex LH-20 and silica gel Lobar C₈ columns were unsuccessful. Separation using an Ito multi-layer

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[‡]Dedicated also to Diane Middlebrook Djerassi (1939–2007), a great humanities scholar.

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coil countercurrent separator was undertaken, with 9:1 CH₃OH–H₂O as the stationary phase and hexane–CH₂Cl₂ as the mobile phase. While this did not provide resolution of the active components, it allowed removal of the more polar impurities and yielded an active 0.88-g fraction suitable for small-scale preparative HPLC, which led to the isolation of two crystalline components that significantly inhibited cancer cell growth, designated bacillistatins 1 (**1**, 34.0 mg) and 2 (**2**, 20.1 mg). Table 1 shows that **1** and **2** have molecular weights a little higher than that of the antibiotic valinomycin (**3**).

By X-ray crystallographic analysis of **1** (Figure 1), its structure was shown to be a 36-membered cyclodepsipeptide that incorporates *R*-valine (*R*-Val), *S*-lactic acid, (*S*-Lac), *S*-valine (*S*-Val), and 2*R*-hydroxy-3*S*-methyl-valeric acid (2*R*-Hy-3*S*-Me-v) and that closely resembles **3**, which consists of three repeating sequences of *R*-Val, *S*-Lac, *S*-Val, and 2*R*-hydroxyisovaleric acid. However, initial attempts at X-ray crystal structure analysis of **2** did not give unequivocal results, and a chemical degradation of each compound was carried out in order to elucidate the structures.

As outlined in Scheme 1, **1** and **2** were subjected to base hydrolyses,^{5a} and the products were analyzed by HPLC-MS (Table 2). Hydrolysis of **1** gave a product that has the same molecular weight and HPLC retention time as the *S*-Lac–*S*-Val (**4**) released by hydrolysis of **3**, along with a later peak that has the correct molecular weight for the 2*R*-hydroxy-3*S*-methyl-valeric acid–*R*-valine condensation product **5**. Thus, the components of the repeating units in **1** were identified and found to be consistent with the X-ray crystal structure.

Hydrolysis of **2** gave the same two products and HPLC peaks as were observed after hydrolysis of **1**, and in addition there was a third peak that has the same molecular weight as **5** but a slightly shorter retention time (Table 3). The ratio of the peaks corresponding to these isomers was approximately 1:2, suggesting that one of the three units in **2** was somewhat different. When the base hydrolyses of **1** and **2** were followed by acid hydrolyses and derivatization with Marfey's reagent (1-fluoro-2,4-dinitrophenyl-5-L-alanine amide; FDAA), only the *S*-Val and *R*-Val derivatives were identified by HPLC (Scheme 1; Table 4). Therefore, the difference between the two bacillistatins was shown to lie in the hydroxy acid moiety. Comparison of the high-field ¹³C NMR spectra of **1** and **2** also supported this location for the difference between these closely related cyclodepsipeptides (Table 5), suggesting the replacement of one of the 3*S*-methyl-valeric acid moieties in **1** by a 4-methyl-valeric acid (4-Me-v) in **2**. The signal at δ 26.15 in the ¹³C NMR spectrum of **1** was assigned to the 6b-, 18b-, and 30b-methylenes of the 3*S*-methyl-valeric acid, whereas the signal at δ 40.79 in the spectrum of **2** was assigned to the 30a-methylene of the 4-methyl-valeric acid; these signals corresponded closely with calculated values, and APT experiments confirmed the assignments. In general, the symmetry of **1** resulted in a simplified ¹³C NMR spectrum with perfect overlap of the signals, whereas **2** gave rise to a complex spectrum with discrete signals for each carbon atom.

To confirm the identity of the hydroxy acid–valine moiety in **2**, a synthetic sample of 2*R*-hydroxy-4-methyl-valeryl-*R*-Val (**6**) was prepared and found to have the same HPLC retention time as the minor product from base hydrolysis of **2** (Table 3). Compound **5** was also prepared as a reference, and Tables 6 and 7 give the ¹H and ¹³C NMR data for **5** and **6** and the intermediate benzyl esters. As in macrocycle **2**, the methylene groups at C-6 (in **5**) and at C-5 (in **6**), as well as the neighboring methyl groups, gave rise to diagnostic peaks in the ¹³C NMR spectrum. The other isomers (**7**–**12**) were prepared in parallel experiments and eliminated as possibilities by HPLC analysis. Bacillistatin 2 (**2**) was therefore determined to comprise two units of *S*-Lac–*S*-Val–2*R*-hydroxy-3*S*-methyl-valeryl–*R*-Val and a third unit in which a 4-methyl-valeric acid replaces the 3*S*-methyl-valeric acid. Structure **2** has now been synthesized, as reported in the accompanying paper,⁷ and its identity with the natural product confirmed.

In broth microdilution susceptibility assays,⁸ **1** and **2** were active against *Streptococcus pneumoniae* and *S. pyogenes* (Table 8). Minimum bactericidal concentrations (MBCs) for *S. pneumoniae* were equal to, or a twofold dilution higher than, the minimum inhibitory concentrations (MICs), indicating that **1** and **2** are bactericidal for *S. pneumoniae*. When broth microdilution assays with *S. pneumoniae* and *S. pyogenes* were performed in the presence of 25% heat-inactivated human serum, MICs were > 64 µg/mL.

Bacillistatins **1** and **2** were more inhibitory than **3** against a human cancer cell-line panel (Table 9) and because of their outstanding activity are being further evaluated, as are methods for biosynthesis and organic synthesis⁷ that will allow structural modification. The remaining components of the original extract are also being further investigated.

Experimental Section

General Experimental Procedures

Solvents used for the chromatographic procedure were redistilled. Sephadex LH-20 employed for gel permeation and partition chromatography was obtained from Pharmacia Fine Chemicals AB, Uppsala, Sweden. The silica gel GHLF Uniplates for thin-layer chromatography were supplied by Analtech, Inc., Newark, DE. The TLC results were viewed under UV light and developed with ceric sulfate–sulfuric acid (heating for 3 min). Reversed-phase HPLC experiments were performed on Luna C₈ (250 × 10 mm, 5 µm), Zorbax C₁₈ (250 × 4.6 mm, 5 µm), and Discovery C₈ (250 × 4.6 or 10 mm, 5 µm) columns attached to Gilson HPLC, Agilent HP1100, or Walters Delta-600 equipment, monitored with UV and ELSD detectors. Low-pressure silica chromatography was carried out on Lobar (B) columns (E. Merck) in 2-propanol–hexane (1:39) and monitored with an ISCO zinc-lamp 214-nm detector. The optical rotation data were determined with a Perkin-Elmer 241 polarimeter. UV spectra were from a Perkin-Elmer Lambda 3β UV/vis spectrophotometer equipped with a Hewlett-Packard Laser Jet 2000 plotter. IR spectra were recorded on an Avatar 360 FT-IR instrument with a single-reflection HATR sample accessory. The NMR experiments were conducted with a Varian Unity INOVA-500 spectrometer operating at 500 MHz and 125 MHz for ¹H NMR and ¹³C NMR spectroscopy, respectively, with use of Shigemi sample tubes and tetramethylsilane as reference. High-resolution mass spectra were obtained on JEOL LCmate or GCmate instruments with poly(ethylene glycol) (PEG) as the reference standard. X-ray structure analyses were performed on a Bruker AXS Smart 6000 diffractometer.

Specimen Collection and Fermentation

A marine crab collected in 1998 near the port town of Quellon on Chiloé Island was rinsed in sterile water, ground with a sterile mortar and pestle, and plated on solid media containing autoclaved local seawater. Pure cultures were shipped to our laboratory for screening. A bacterial isolate from the crab with significant human cancer cell line activity was identified by 16S rRNA sequencing (Acculab, Newark, DE) as *Bacillus silvestris* (% difference = 0.09; confidence level to species). *Bacillus silvestris* was scaled up to 378 L at 125 rpm for six days at room temperature in an aqueous medium containing dextrose (0.5 g/L), yeast extract (1.25 g/L), peptone (2.5 g/L), and instant ocean (26.5 g/L).

Extraction and Solvent Partition of *Bacillus silvestris*

The bacterial broth was extracted with CH₂Cl₂, and the extract was concentrated to a dark oil that was redissolved in CH₃OH–H₂O (3:2). The aqueous solution was successively extracted with hexane and CH₂Cl₂, and solvent was removed from both extracts and from the remaining aqueous solution. The CH₂Cl₂-soluble (4.65 g; P388: ED₅₀ 0.01 µg/mL) and the CH₃OH–H₂O-soluble (0.8 g; P388: ED₅₀ 0.24 µg/mL) extracts were retained. The hexane-soluble extract (44.2 g; P388: ED₅₀ 0.02 µg/mL) was partitioned between hexane and acetonitrile to

give a hexane-soluble yellow oil (26.8 g; P388: ED₅₀ 0.08 µg/mL) and an acetonitrile-soluble viscous gum (15.9 g; P388: ED₅₀ 0.03 µg/mL). An 11-g aliquot of the acetonitrile-soluble extract was dissolved in 9:1 CH₃OH–H₂O, and this solution was extracted (5 ×) with 9:1 hexane–CH₂Cl₂. The CH₃OH–H₂O solution was then concentrated to a dark-brown gum (3.14 g; P388: ED₅₀ 0.007 µg/mL).

Isolation of Bacillistatins 1 and 2

The cancer cell growth inhibitory CH₃OH–H₂O fraction (3.14 g), obtained as described above, was shown by HPLC [Zorbax SB C₈ column (150 × 4.6 mm); 19:1 acetonitrile–0.05 M acetic acid, 40 °C, 2 mL/min] to contain at least six closely spaced and late-eluting compounds. Fractionation on an Ito multi-layer coil countercurrent separator was undertaken, with 9:1 CH₃OH–H₂O as the stationary phase and hexane–CH₂Cl₂ as the mobile phase, which allowed removal of the polar impurities (1.56 g). From the remaining 0.88-g bioactive mixture, 25-mg aliquots were fractionated by semi-preparative HPLC on a Hewlett-Packard HP-1100 LC with a Luna C₈ column (Phenomenex; 250 × 10 mm) in 9:1 CH₃OH–0.05 M acetic acid (isocratic; 3.5 mL/min). The column effluent was monitored at 235 nm, and about 10% of the flow was diverted by a splitter valve (Upchurch P-451) to an ELSD detector (Sedex-55), because the sample did not absorb strongly except at lower wavelengths. Cyclodepsipeptides **1** (34.0 mg) and **2** (20.1 mg) were obtained by pooling and concentrating the fractions that showed peaks at retention times of 52.40 and 44.97 min, respectively, from several runs.

Bacillistatin 1 (1)

Colorless prisms from CH₃OH–H₂O; ¹H NMR (DMSO) δ 0.80–0.95 (54 H, m, 18 × CH₃), 1.14–1.26 (9 H, m, CH₃-12a, -24a, -36a), 1.26–1.37 (6 H, m, CH₂-6b, -18b, -30b), 1.90–1.96 (3 H, m, CH-6a, -18a, -30a), 2.12–2.22 (6 H, m, CH-3a, -9a, -15a, -21a, -27a, -33a), 4.24 (3 H, t, *J* = 8.0 Hz, CH-9, -21, -33), 4.50 (3 H, t, *J* = 7.2 Hz, CH-3, -15, -27), 4.94 (3 H, d, *J* = 3.2 Hz, CH-6, -18, -30), 5.04 (3 H, q, *J* = 6.4 Hz, CH-12, -24, -36), 7.86 (3 H, d, *J* = 8.0 Hz, NH-4, -16, -28), 8.43 (3 H, d, *J* = 8.0 Hz, NH-10, -22, -34); ¹³C NMR data, see Table 5; HRMS (APCI +) *m/z* 1153.6881 [M+H]⁺ (calcd for C₅₇H₉₇N₆O₁₈, 1153.68594).

X-ray Crystal Structure Determination (1)

A plate-shaped crystal (~ 0.32 × 0.29 × 0.11 mm), grown from a CH₃OH–H₂O solution, was mounted on the tip of a glass fiber. Cell parameter measurements were taken and data collected at 153 ± 1 K with a Bruker SMART 6000 diffractometer system using Cu K radiation. A sphere of reciprocal shape was covered by use of the Multirun technique.⁹ Thus, six frames of data were collected with 0.396° steps in ω and a seventh set with 0.396° steps in ϕ so that 98.5% coverage of all unique reflections to a resolution of 0.822 Å was accomplished.

Crystal Data

C₅₇H₉₆N₆O₁₈, FW = 1153.40, rhombohedral, *R*3, *a* = *b* = *c* = 26.6254(4) Å, α = β = γ = 118.0470 (10)°, *V* = 6775.05(18) Å³, *Z* = 4, ρ_c = 1.131 mg/m³, μ (Cu K α) = 0.690 mm⁻¹, λ = 1.54178 Å.

A total of 48 578 reflections was collected, of which 14 573 reflections were independent reflections (*R*(int) = 0.0661). Subsequent statistical analysis of the data set with the XPREP¹⁰ program indicated the rather uncommon spacegroup *R*3. Final cell constants were determined from the set of the 8732 observed (>2 σ (*I*)) reflections. An absorption correction was also applied to the data with the Bruker program SADABS.¹¹ The structure solution and refinement was readily accomplished with the direct-methods program SHELTXL^{12,13} using 8732 (>2 σ (*I*)) observed reflections. All non-hydrogen atom coordinates were located in a routine run using default values for that program. The remaining H atom coordinates were calculated at optimum positions with that program, and all non-hydrogen atoms were then

refined anisotropically in a full-matrix least-squares refinement procedure. The H atoms were included, their U_{iso} thermal parameters fixed at either 1.2 or 1.5 (depending on the atom type) of the value of the U_{iso} of the atom to which they were attached, and forced to ride that atom. The final residual R_1 value for the model shown in Figure 2 was 0.0959 for observed data and 0.1233 for all data. The goodness-of-fit on F^2 was 0.959. The corresponding Sheldrick R values were wR_2 of 0.2292 and 0.2472, respectively. The final model for **1** is shown in Figure 1 and is composed of three repeating peptide units, each of which contains the following sequence: *R*-Val, *S*-Lac, *S*-Val, and 2*R*-hydroxy-3*S*-methyl-valeric acid. The asymmetric unit cell of **1** was found to contain one complete 36-membered macrocyclic depsipeptide ring and an additional monomeric unit consisting of *R*-Val-*S*-Lac-*S*-Val-2*R*-Hy-3*S*-Me-v, which is one-third of another molecule in an adjoining asymmetric cell unit. As a consequence, the unit cell contains four bacillistatin 1 molecules. A final difference Fourier map showed some residual electron density, the largest difference peak and hole being 1.147 and $-0.496 \text{ e}/\text{\AA}^3$, respectively. However, all residual peaks were attributed to local disorder of the non-hydrogen atoms already assigned. Final bond distances and angles were all within expected and acceptable limits. The Flack absolute structure parameter χ for the model shown in Figure 1 was 0.2(3), indicating that the absolute configuration depicted is the correct stereoisomer of **1**.

Bacillistatin 2 (2)

Colorless prisms from $\text{CH}_3\text{OH}-\text{H}_2\text{O}$; ^1H NMR (DMSO) δ 0.79–0.97 (54 H, m, $18 \times \text{CH}_3$), 1.14–1.26 (9 H, m, CH_3 -12a, -24a, -36a), 1.26–1.36 (4 H, m, CH_2 -6b, -18b), 1.46–1.58 (1 H, m, H_a -30a), 1.58–1.75 (1 H, m, H_b -30a), 1.88–2.00 (3 H, m, CH -6a, -18a, -30a), 2.12–2.24 (6 H, m, CH -3a, -9a, -15a, -21a, -27a, -33a), 4.18–4.30 (3 H, m, CH -9, -21, -33), 4.38–4.45 (2 H, m, CH -3, -15), 4.45–4.54 (1 H, m, CH -27), 4.93 (3 H, m, CH -6, -18, -30), 5.04 (3 H, m, CH -12, -24, -36), 7.90 (2 H, d, $J = 8.0 \text{ Hz}$, NH -4, -16), 7.96 (1 H, d, $J = 8.0 \text{ Hz}$, NH -28), 8.38 (3 H, d, $J = 8.0 \text{ Hz}$, NH -10, -22, -34); ^{13}C NMR data, see Table 5; HRMS (APCI+) m/z 1153.6885 [$\text{M} + \text{H}$] $^+$ (calcd for $\text{C}_{57}\text{H}_{97}\text{N}_6\text{O}_{18}$, 1153.68594).

Base Hydrolysis of Bacillistatin 1 (1) ^{5a}

To a solution of **1** (100 μg) in CH_3OH (100 μL) in a cone-bottomed vial was added an aqueous solution of KOH (1.2 *N*; 100 μL), and the mixture was heated at 50 $^\circ\text{C}$ for 1.5 h before being cooled and neutralized with aqueous HCl (6 *N*; 20 μL). The resulting mixture was dried under nitrogen, with final drying under high vacuum, and the residue was dissolved in water (100 μL). Only valine (*R* or *S*) was detected when a 2- μL aliquot of the aqueous solution was analyzed for amino acid content. The remainder of the product solution was investigated by LC-MS: a HPLC peak for one of the products had the same retention time as that of **4**, which was generated by hydrolysis of **3**, and a later peak had the correct molecular weight for a 2-hydroxy-3-methyl-valeryl-valine isomer with a configuration shown by the X-ray analysis above to be 2*R*-hydroxy-3*S*-methyl-valeryl-*R*-valine (**5**).

Base Hydrolysis of Bacillistatin 2 (2)

The same procedure was followed as described above for **1**. Amino acid analysis of an aqueous solution of the product detected valine, and HPLC analysis showed the same two peaks (corresponding to **4** and **5**) as were generated by hydrolysis of **1**. A third peak was also observed, with the same molecular formula as **5** but with a slightly longer retention time and in about half the concentration of **5**.

Determination of the Valine Configuration in 1 and 2 ^{5a}

The preceding base hydrolyses were repeated with 1.2 *N* KOH on 100- μg samples of **1**, **2** and **3**. The products were next treated with 6 *N* HCl (100 μL) in 1-mL cone-bottomed vials with

Teflon-lined screw caps, and the mixtures were heated overnight at 110 °C. The vials were then cooled and the liquid contents transferred by syringe to similar vials prior to being dried in a vacuum oven. The crude hydrolysates were redissolved in water before being treated with a solution of 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA, Marfey's reagent; 1% in acetone; 50 μ L), followed by NaHCO₃ (1 M; 10 μ L). The tubes containing the resultant mixtures were placed in a block heater for 1.5 h at 45 °C, and the mixtures were then cooled, neutralized with 2 N HCl (5 μ L), and diluted with CH₃OH (500 μ L) for HPLC. The derivatives were separated by chromatography on a Zorbax SB C₁₈ column in a gradient of 20–50% acetonitrile in aqueous TFA (0.1%) at 1.0 mL/min for 30 min. The column was monitored at 340 nm and by ELSD. Reference standards of DNPA-amino acids were also prepared from *R*-valine and *S*-valine. This procedure established the presence of both *R*-Val and *S*-Val in each cyclodepsipeptide (Table 4).

Illustrative Procedure for Synthesis of Chiral Isomers 5–12: Preparation of 6. 2*R*-Hydroxy-4-methyl-valeric acid ¹⁴

To a solution of D-leucine (0.5 g) in aqueous perchloric acid (0.5 N; 200 mL) that was cooled to < 5 °C and stirred vigorously was added a solution of sodium nitrite (7 g) in ice-cold water (50 mL). Stirring was continued for 30 min with warming to room temperature, and the mixture was then heated to reflux under a current of nitrogen until evolution of N₂O₄ had ceased. The mixture was cooled to room temperature, saturated with sodium chloride, and extracted once with ethyl acetate (50 mL). The organic layer was dried over MgSO₄, and removal of solvent yielded a pale yellow oil with suspended colorless solids. The residue was taken up in a small volume of CH₂Cl₂ and the solids were collected; the filtrate was dried under nitrogen, with final drying in a vacuum oven, to give 2*R*-hydroxy-4-methyl-valeric acid as a clear oil (0.371 g).

R-Val Benzyl Ester

To a suspension of *R*-Val-OBz *p*-toluenesulfonate (0.76 g) in H₂O (5 mL) was added saturated aqueous NaHCO₃ (340 mg in 5 mL). The mixture was vigorously sonicated until the suspension had become a gelatinous semisolid, and it was then extracted with CH₂Cl₂ (4 $\times$ 5 mL). The combined organic phase was dried over MgSO₄, and removal of solvent yielded *R*-Val-OBz as a clear oil that crystallized on standing (0.41 g).

2*R*-Hydroxy-4-methyl-valeryl-*R*-valine (6)

To *R*-Val-OBz (0.41 g) was added a solution of 2*R*-hydroxy-4-methyl-valeric acid (0.37 g) in CH₂Cl₂ (10 mL), followed by a solution of dicyclohexylcarbodiimide (DCCI; 0.41 g) in CH₂Cl₂ (5 mL). The mixture was stirred at room temperature for 4.5 h and then retained at 5 °C for 48 h. The precipitated dicyclohexylurea was collected, and the filtrate was washed successively with saturated aqueous NaHCO₃, 2 N HCl, and H₂O. The organic phase was dried over MgSO₄ and the solvent was removed (with final drying in a vacuum oven) to yield a pale yellow gum (0.76 g), which was fractionated by chromatography on a prepacked silica column (Ace Glass, Inc.) in hexane–2-propanol (39:1) at 6 mL/min to give several products, including 2*R*-hydroxy-4-methylvaleryl-*R*-valine benzyl ester: HRMS (APCI+) *m/z* 322.1989 [M+H]⁺ (calcd for C₁₈H₂₈NO₄, 322.2018). Removal of the benzyl group was carried out as follows: to a solution of the benzyl ester (70 mg) in a mixture of ethanol–H₂O–acetic acid (5 mL; 7:2:1) was added 10% palladium-on-charcoal (25 mg). The mixture was stirred under hydrogen for 3 h, and the solvent was filtered. Removal of solvent from the filtrate yielded **6** as a colorless oil (46.9 mg): HRMS (APCI+) *m/z* 232.1533 [M+H]⁺ (calcd for C₁₁H₂₂NO₄, 232.1549). For ¹H and ¹³C NMR data, see Tables 6 and 7.

Human Cancer and Murine Lymphocytic Leukemia Cell Line Procedures

The inhibition of human cancer cell growth was determined with the National Cancer Institute's standard sulforhodamine B assay as earlier described.¹⁵ In summary, cells in a 5% fetal bovine serum/RPMI-1640 medium solution were inoculated in 96-well plates and incubated for 24 h. Serial dilutions of the compounds were then added. After 48 h, the plates were fixed with trichloroacetic acid, stained with sulforhodamine B, and read with an automated microplate reader. A growth inhibition of 50% (GI₅₀ or the drug concentration causing a 50% reduction in the net protein increase) was calculated from optical density data with Immunosoft software.

Mouse lymphocytic leukemia P388 cells¹⁶ were incubated for 24 h in a 10% horse serum/Fisher medium solution followed by a 48-h incubation with serial dilutions of the compounds. Cell growth inhibition (ED₅₀) was calculated with a Z1 Beckman/Coulter particle counter.

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Figure 1.
X-ray structure (excluding hydrogens) of bacillistatin 1 (**1**), depicted as 50% probability thermal ellipsoids.

Scheme 1.
Chemical Degradation of **1** and **2**

1, bacillistatin 1 **2, bacillistatin 2**

3, valinomycin **4**, S-Lac-S-Val **5**, 2*R*-hydroxy-3*S*-methylvalery-*R*-Val **6**, α -OH, 2*R*-hydroxy-4-methylvalery-*R*-Val**7**, β -OH **8**, α -CH₃, β -OH**9**, β -CH₃, α -OH**10**, β -CH₃, β -OH **11**, α -CH₃, β -OH**12**, β -CH₃, α -OH

Table 1
LC/MS Data for *B. sylvestris* Extract and Valinomycin (**3**)

peak (min)	compound	[M + H] ⁺ (m/z)	molecular formula	error (ppm)
32.30		1125.663	C ₅₅ H ₉₂ N ₆ O ₁₈	7.4
32.92		1125.662	C ₅₅ H ₉₂ N ₆ O ₁₈	6.5
32.92		1139.679	C ₅₆ H ₉₄ N ₆ O ₁₈	7.6
33.65		1139.668	C ₅₆ H ₉₄ N ₆ O ₁₈	-2
33.65		1153.686	C ₅₇ H ₉₆ N ₆ O ₁₈	0
34.37		1139.660	C ₅₆ H ₉₄ N ₆ O ₁₈	-9.1
34.37		1153.690	C ₅₇ H ₉₆ N ₆ O ₁₈	3.5
35.21	2	1153.699	C ₅₇ H ₉₆ N ₆ O ₁₈	11
36.11	1	1153.697	C ₅₇ H ₉₆ N ₆ O ₁₈	9.6
reference	3	1111.640	C ₅₄ H ₉₀ N ₆ O ₁₈	

Table 2
LC-MS Identification of Products of Hydrolyses of **1** and **2**^a

substrate	peak	[M + H] ⁺ (<i>m/z</i>)	molecular formula	product
1	1	190.1172	C ₈ H ₁₆ NO ₄	4
1	2	232.1499	C ₁₁ H ₂₂ NO ₄	5
2	1	190.1163	C ₈ H ₁₆ NO ₄	4
2	2	232.1499	C ₁₁ H ₂₂ NO ₄	6
2	3	232.1502	C ₁₁ H ₂₂ NO ₄	5

^aHPLC conditions: Zorbax SB C₁₈ column (250 mm × 4.6 mm); 10% to 40% acetonitrile in 0.1% aq. TFA at 1.0 mL/min for 30 min.

Table 3
HPLC Comparison of Bacillistatin Hydrolyses Products^a

substrate	hydrolysis product <i>or</i> reference	retention time (min)
reference	5	22.02
reference	6	21.34
2 (peak 2) ^b	6	21.38
2 (peak 3) ^b	5	22.05
1 (peak 2) ^b	5	22.05

^aHPLC conditions: Zorbax SB C₁₈ column (250 mm × 4.6 mm); 10% to 30% acetonitrile in 0.1% aq. TFA at 1.5 mL/min for 30 min; ELSD detection.

^bSee Table 2.

Table 4

Retention Time of the DNPA-Amino Acids^a following Marfey's Derivatization of the Products of Hydrolyses of **1**, **2** and **3**^b

substrate	DNPA-S-Val (min)	DNPA-R-Val (min)
1	16.775	19.600
2	16.815	19.666
3	16.835	19.638

^a2,4-Dinitrophenyl-5S-alaninamide amino acid.

^bHPLC conditions: Zorbax SB C₁₈ column (250 mm × 4.6 mm); gradient of 10% to 40% acetonitrile in 0.1% aq. TFA at 1.0 mL/min for 30 min.

Table 5¹³C NMR Spectroscopic Assignments for Bacillistatins 1 (**1**) and 2 (**2**) ^a

1		2	
position	δ_c, mult.	Position	δ_c, mult.
6c, 18c, 30c	11.74, CH ₃	6c, 18c	11.77, CH ₃
6d, 18d, 30d	14.23, CH ₃	6d, 18d	14.27, CH ₃
12a, 24a, 36a	17.06, CH ₃	12a, 24a	16.92, 17.12, CH ₃
3b, 15b, 27b ^b	18.99, CH ₃	36a	17.27, CH ₃
3c, 15c, 27c ^b	19.22, CH ₃	3b, 3c, 9b, 9c, 15b, 15c, 21b, 21c, 27b, 27c, 33b, 33c	18.97, 19.17, 19.21, 19.27, 19.39, 19.46, 19.58, 19.81, 19.86, CH ₃
9b, 21b, 33b ^c	19.46, CH ₃	30c	21.49, CH ₃
9c, 21c, 33c ^c	19.64, CH ₃	30d	23.19, CH ₃
6b, 18b, 30b ^d	26.15, CH ₂	30b	24.51, CH
		6b, 18b ^d	26.20, 26.33, CH ₂
9a, 21a, 33a	28.36, CH	9a, 21a	28.30, 28.36, CH
3a, 15a, 27a	28.47, CH	3a, 15a	28.41, 28.43, CH
		27a	28.46, CH
		33a	28.70, CH
6a, 18a, 30a	36.84, CH	6a, 18a	36.77, 36.88, CH
		30a ^d	40.79, CH ₂
		27	58.51, CH
3, 15, 27	58.77, CH	3, 15, CH	59.10, 59.16
		33	60.85
9, 21, 33	60.32, CH	9, 21	60.95, CH
		36	70.18, CH
12, 24, 36	70.35, CH	12, 24	70.54, 70.62, CH
		30	73.39, CH
6, 18, 30	77.32, CH	6, 18	76.60, CH
8, 20, 32	169.98, CO	2, 5, 8, 11, 14, 17, 20, 23, 26, 29, 32, 35	169.89, 170.21, 170.28, 170.88, 170.94, 171.51, 171.70, 171.87, 172.17, 172.27, 172.30, 172.61, CO
2, 14, 26	170.21, CO		
11, 23, 35	171.64, CO		
5, 17, 29	172.41, CO		

^aRecorded in CD₃OD^bAssignments may be reversed.^cAssignments may be reversed.^dAssigned via APT.

Table 6

¹H NMR Spectroscopic Assignments for Carboxylic Acids **5** and **6** and the Corresponding Benzyl Esters (recorded in CDCl₃)

position	5 δ _H , mult.	6 δ _H , mult.	5 , benzyl ester δ _H , mult.	6 , benzyl ester δ _H , mult.
2b, 2c, 5a, 7	0.820–0.998		0.824–0.971	
2b, 2c, 6a, 7		0.916–0.976		0.853–0.963
6-H _a	1.260–1.392, m		1.262–1.381, m	
6-H _b	1.434–1.562, m		1.416–1.533, m	
5-H _a , 5-H _b		1.489–1.617, m		1.487–1.679, m
6		1.765–1.948, m		1.795–1.908, m
5	1.815–1.920, m		1.860–1.980, m	
2a	2.160–2.300, m	2.165–2.330, m	2.170–2.278, m	2.119–2.265, m
OH	3.311		2.992	3.443
4	4.043–4.062, m	4.181–4.223, m	4.157, s	4.155–4.186, m
2	4.372–4.399, m	4.421–4.448, m	4.598–4.644, m	4.569–4.615, m
8			5.109–5.228, m	5.098–5.223, m
NH/OH	4.91	7.395, 7.424	7.072–7.102	7.098, 7.127
phenyl			7.351	7.345

Table 7¹³C NMR Spectroscopic Assignments for Carboxylic Acids **5** and **6**

position	5 ^a δ _c , mult.	6 ^b δ _c , mult.
1	173.42, CO	173.47, CO
2	56.37, CH	56.83, CH
2a	30.32, CH	30.99, CH
2b	19.12, CH ₃	19.65, CH ₃
2c	17.71, CH ₃	18.28, CH ₃
3	173.10, CO	174.99, CO
4	72.96, CH	70.21, CH
5	37.69, CH	44.27, CH ₂
5a	13.24, CH ₃	
6	25.77, CH ₂	24.56, CH
6a		24.09, CH ₃
7	11.83, CH ₃	22.11, CH ₃

^aRecorded in CDCl₃.^bRecorded in DMSO.

Table 8

Minimum Inhibitory Concentrations (MIC) and Minimum Bactericidal Concentrations (MBC) of Bacillistatins 1 (**1**) and 2 (**2**)^a

clinical isolate or (reference strain)	MIC (µg/mL)/MBC (µg/mL)	
	1	2
<i>Streptococcus pneumoniae</i> (ATCC 6303)	2/4	½
penicillin-resistant <i>S. pneumoniae</i>	1/2	1/1
multidrug-resistant <i>S. pneumoniae</i> (ATCC 700673)	<0.5/1	<0.5/<0.5
<i>S. pyogenes</i>	2/ND	8/>64
<i>S. pyogenes</i>	4/>32	2/>16

^a Against *Cryptococcus neoformans* (ATCC 90112), *Candida albicans* (ATCC 90028), *Staphylococcus aureus* (ATCC 29213), *Enterococcus faecalis* (ATCC 29212), *Micrococcus luteus* (Presque Isle 456), *Escherichia coli* (ATCC 25922), *Enterobacter cloacae* (ATCC 13047), *Stenotrophomonas maltophilia* (ATCC 13637), and *Neisseria gonorrhoeae* (ATCC 49226) there was no inhibition at 64 µg/mL.

Table 9

Inhibition of the Murine P388 Lymphocytic Leukemia (ED₅₀ µg/mL) and Human Cancer Cell Lines (GI₅₀ µg/mL) by Bacillistatins 1 (1) and 2 (2), with Valinomycin (3) as Reference^a

compound	P388	BXPC-3	MCF-7	cell line ^b SF-268	NCI-H460	KM20L2	DU145
1	0.023	0.00095	0.00061	0.00045	0.00230	0.00087	0.00150
2	0.013	0.00034	0.00031	0.00180	0.00045	0.00026	0.00086
3	0.120	0.0019	0.0010	0.0027	0.0025	0.0008	0.0035

^aDMSO was used as vehicle in the testing.

^bCancer cell lines in order: murine lymphocytic leukemia (P388); pancreas (BXPC-3); breast (MCF-7); CNS (SF-268); lung (NCI-H460); colon (KM20L2); prostate (DU-145).