Isolation and Structure of Stylostatin 1 from the Papua New Guinea Marine Sponge *Stylotella aurantium*[1a]

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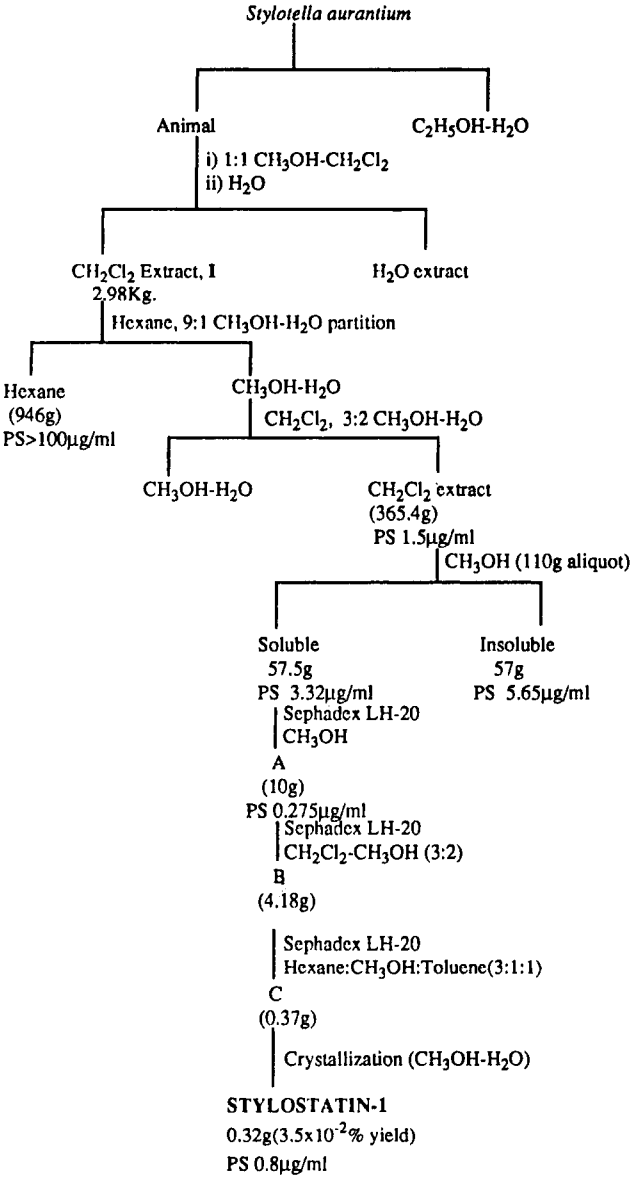
A new cell growth inhibitory (P388 lymphocytic leukemia ED_{50} 0.8 μg/mL) cycloheptapeptide designated stylostatin 1 (1) has been isolated from the south Pacific Ocean sponge *Stylotella aurantium* Kelly-Borges and Bergquist. Structural determination was accomplished primarily by utilizing high-field ^{1}H-NMR (500 MHz) with appropriate 2D-NMR experiments and was confirmed by X-ray crystallographic analysis. The absolute configuration (established by chiral gas chromatographic analysis) and crystal structure conformation (with hydrogen bonding) for stylostatin 1 (1) were determined.

While the isolation of cytotoxic marine sponge constituents has been rapidly increasing[2] only a few of these compounds have represented cyclic peptide[2a,b,3] and protein classes.[4] Previously, we described discovery of the cell growth inhibitory cycloheptapeptide axinastatin 1[2a] and the cyclooctapeptide hymenistatin 1[3] in Western Caroline Island (Palau) Porifera. Continued investigation of tropical marine sponges for antineoplastic and/or cytotoxic components has now resulted in isolation and structural elucidation of a new cytostatic cycloheptapeptide herein named stylostatin 1 from the Papua New Guinea marine sponge *Stylotella aurantium* Kelly-Borges and Bergquist. The discovery of stylostatin 1 and its assignment as cyclo[Leu-Ala-Ile-Pro-Phe-Asn-Ser] will now be described.

On our second[5] expedition to Papua New Guinea in 1983, we explored a variety of marine habitats northwest of New Ireland (in the Bismark Archipelago) between the south Pacific Ocean and the Bismark Sea. An orange sponge with a yellowish interior that occurs in erect fused finger-type shapes, later identified as *Stylotella aurantium* Kelly-Borges and Bergquist (order Hadromerida, class Demospongiae), was found to provide an ethanol extract which showed promising results against the U. S. National Cancer Institute's murine P388 lymphocytic leukemia (PS system) with 44–68% increases in life span at 50–400 mg/kg. A 500-kg (wet wt) recollection of *S. aurantium* Kelly-Borges and Bergquist was completed in 1986.

Scheme I



After extraction and preliminary separation by initial solvent partitioning (Scheme I) the sponge gave a PS active methylene chloride extract that was successively partitioned between 9:1 → 3:2 methanol–water with hexane → methylene chloride. Upon evaporation of the chlorocarbon solvent a brown oil was obtained which significantly inhibited (ED_{50} 1.5 μg/mL) the PS leukemia cell line. Initial

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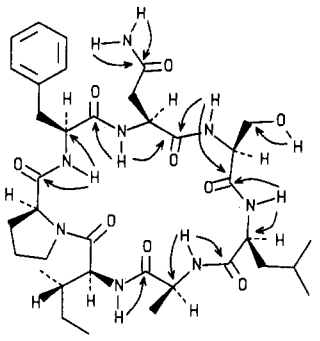

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Figure 1. Stylostatin 1 with some important HMBC correlations.

gel-permeation chromatographic separation on a column of Sephadex LH-20 in methanol was followed by another LH-20 chromatography of the resulting active fraction with 3:2 methylene chloride–methanol as eluent yielding a concentration of activity. Further separation was achieved by employing column partition chromatography on Sephadex LH-20 with 3:1:1 hexane–toluene–methanol as the mobile phase. The light-yellow solid obtained from this separation step was repeatedly crystallized from methanol–water to afford the cell growth inhibitory (PS ED_{50} 0.8 μg/mL) biosynthetic product designated stylostatin 1 (1) as a pure colorless crystalline solid (Scheme I).

Stylostatin 1 exhibited a high-resolution mass spectral molecular ion peak at m/z 743.408 80, corresponding to molecular formula $C_{36}H_{54}H_8O_9$. Amino acid analyses of this peptide indicated the presence of seven amino acids, phenylalanine, proline, isoleucine, alanine, leucine, serine, and aspartic acid/asparagine, all present in equal amounts. The presence of eight carbonyl signals in the ^{13}C-NMR spectrum supported this assignment. The high intensity of the molecular ion peak in the mass spectrum indicated that the presumed heptapeptide had a cyclic structure. The presence of the aforementioned amino acids was also confirmed by 2D-NMR, ^{1}H–^{1}H-COSY, HMQC,[6] and HMBC[7] experiments. The presence of asparagine rather than aspartic acid was further confirmed by the HMBC experiment. Both the amide protons showed long-range couplings to the carbonyl signal at δ 172.5 (C-4 of asparagine). Additional evidence concerning the amino acid composition was obtained from the mass spectral fragmentation which exhibited a strong peak corresponding to a loss of 17 units (ammonia from Asn). From the HMBC experimental results (Figure 1), the sequence was identified as cyclo[Leu-Ala-Ile-Pro-Phe-Asn-Ser].

The proposed structure for stylostatin 1 (1) was confirmed by single-crystal X-ray crystallography. Stable crystals for X-ray crystallography were difficult to obtain due to the apparent tendency of the compound to form highly solvated and unstable crystal complexes with water and alcohol. However, growing crystals from 2-propanol–water resulted in a specimen sufficiently stable at reduced temperature to provide useful diffraction data. Reflections were collected at −104 °C, along with their Friedel pairs, over one quadrant. Structure solution and refinement revealed a single cyclic peptide molecule associated with at least two water molecules and one molecule of 2-propanol. Residual electron density in the unit cell (highest peak, 1.12 e/Å^3) suggested that other, highly disordered water molecules may also be present. Such an assumption seemed to be confirmed by the variability in

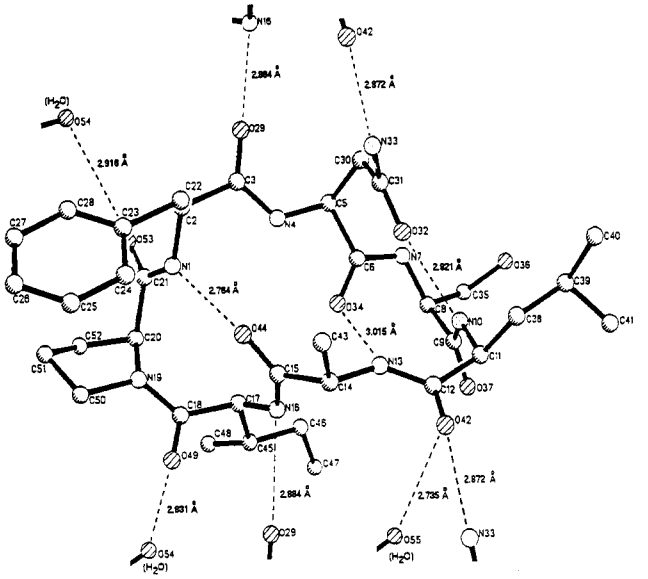

Figure 2. Computer-generated perspective drawing and X-ray numbering system (less hydrogens). Inter- and intramolecular atomic distances of the flanking heteroatoms believed to be involved in hydrogen bonding are also shown.

density measurements which suggested a range of peptide hydration.

A computer-generated perspective drawing depicting the absolute configuration and conformation of the cyclic peptide appears in Figure 2. The absolute configuration was derived from the amino acid chiral analyses noted below. The conformation of stylostatin 1 is such that the phenyl ring from phenylalanine and the pyrrolidine ring from proline are nearly coplanar, one lying above the other. All of the carbonyl oxygens present in stylostatin 1 are apparently involved in hydrogen bonding, either through intramolecular or intermolecular interactions (see Figure 2). Thus, the carbonyl groups containing the O32, O34, and O44 oxygens (X-ray numbering system, Figure 2) are directed toward the center of the macrocyclic peptide ring and are hydrogen bonded to the N10, N13, and N1 amide hydrogens, respectively. The carbonyl groups containing the O29, O42, O49, and O53 oxygens are directed away from the macrocyclic ring and are involved in intermolecular H-bonding with either amide hydrogens from adjacent stylostatin 1 molecules or with hydrate hydrogen atoms. Hydrogen bonding may also be involved with the 2-propanol solvate. However, this possibility could not be verified due to apparent disorder and failure to locate all four non-hydrogen atoms, i.e., only three of the four non-hydrogen atoms of the 2-propanol solvate could be located from difference maps and as such were refined isotropically as carbon atoms.

Since it was only possible to determine the relative stereochemistry from the crystal structure determination it was necessary to ascertain the absolute configuration of the *N*-pentafluoropropionyl isopropyl ester derivatives[8] prepared from the peptide hydrolysate using chiral gas chromatographic analysis. From these experiments, isoleucine present in the peptide was found to be *S* (L). Thus, from the relative stereochemistry obtained from the X-ray

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data, all the amino acids present were assigned the *S* absolute configuration.

The discovery of and unequivocal structure assignment of stylostatin 1 further enhances our understanding of cyclic peptide structural requirements for cell growth inhibitory activity.

Experimental Section

General Methods. Solvents used for chromatographic procedures were redistilled. The Sephadex LH-20 (25–100 μm) employed for gel permeation and partition chromatography was obtained from Pharmacia Fine Chemicals AB, Uppsala, Sweden. The silica gel GF Uniplates for TLC were from Analtech Inc., Newark, DE. All TLC plates were viewed with UV light and/or developed with a ceric sulfate–sulfuric acid spray (heating to approximately 150 °C for 5–10 min). The GC analysis was performed with a Chirasil-Val III FSOT capillary column (50 m × 0.25 mm). The amino acid analyses were conducted with a Hewlett-Packard reversed-phase HPLC column ODS Hypersil C18 (2.1 × 200 mm).

NMR spectra were taken in deuteriodimethylsulfoxide.

Animal Collection and Preliminary Experiments. In 1983, during the Kavieng (New Ireland) segment of our Papua New Guinea expedition, a specimen of the sponge *S. aurantium* Kelly-Borges and Bergquist (order Hadromerida, class Demospongiae) was collected by SCUBA in Byron Strait adjacent to the SE section of Nub Island at a depth of −25 m. Very noteworthy was the observation that this is a remarkable location for large sponges and splendid specimens of other marine fauna.

S. aurantium Kelly-Borges and Bergquist corresponds to an orange sponge with a yellowish interior, which occurs in lobular or flabelliform shapes, measuring up to 8 cm high and 8 cm wide. Taxonomic identification was conducted at the University of Southern California where reference specimens are on file. The specimen differs from that described[9,10] in that the Kavieng sponge is tougher, probably because of the abundance of spongin. The styles are smaller (291–329–370 × 10–14–19 mass units; $n = 10$). This species is common but patchy in distribution at depths of 1–10 m in Madang, Papua New Guinea.[10] The sponge is distributed at Kavieng (New Ireland), Papua New Guinea (north and south coasts), Ponape, Truk Lagoon, and the Palau Islands. The initial ~2 kg (wet wt) sample of *S. aurantium* Kelly-Borges and Bergquist was preserved in ethanol–water. Removal of solvent gave an extract that reached a confirmed level of activity against the U. S. National Cancer Institute's murine P388 lymphocytic leukemia (PS system) exhibiting T/C 144–168 at 50–400 mg/kg.

Animal Extraction and Partitioning. Approximately 500 kg of this sponge was recollected northwest of Kavieng, New Ireland, Papua New Guinea, in July 1986 and preserved in ethanol–sea water. The shipping solution was drained from the barrels and replaced with 1:1 methanol–dichloromethane. The methanol–dichloromethane extract was drained, and 10–20% by volume of water was added to separate the phases. The dichloromethane phase was concentrated to a 2.98-kg residue. The latter was partitioned between hexane and 9:1 methanol–water (~30 L each) to give a 946-g hexane fraction (P388 ED_{50} >100). The interfacial solid was suspended in dichloromethane and added to the methanol–water fraction. Enough water was added to make a 3:2 methanol–water solution followed by 40 L of dichloromethane. The dichloromethane fraction (365 g) was obtained as a brown oil (PS ED_{50} 1.5 μg/mL).

Isolation of Stylostatin 1 (1). A 110-g sample of the preceeding PS active dichloromethane fraction was dissolved in methanol and chromatographed (see Scheme I) on a column of Sephadex LH-20 (120 × 17 cm). Concentration to an active fraction designated A (10 g, PS ED_{50} 0.27 μg/mL) was realized. Next, this active fraction was subjected to partition chromatography on a Sephadex LH-20 column (135 × 10.5 cm) with dichloromethane–methanol (3:2) as eluent to give fraction B (4.18 g). Partition chromatography of fraction B on a Sephadex LH-20 column (225 × 4.5 cm) with 3:1:1 hexane–toluene–methanol as eluent provided active fraction C (0.37 g). Final purification of C was achieved by crystallization from aqueous methanol. Stylostatin 1 was isolated as a colorless crystalline solid (0.32 g 0.032% yield): mp 210 °C (capillary tube); $[\alpha]^{25}_D$ −116° (*c* 0.29, CH_3OH); UV (EtOH) λ_{max} (ϵ) 282 (3087), 231 (5072), 212 (4059), and 208 (4125) nm; HRFAB *m/z* 743.4088, calcd 743.4092 for $C_{36}H_{54}N_8O_9$; IR (KBr) 3453, 3337, 3068, 2965, 2876, 1649, 1557, 1456, 1422, 1311, 1244, 1084, and 702 cm^{-1}; NMR (DMSO-d_6) **serine** unit, ^1H 3.6826 (brt, $J = 5$ Hz, H-3), 3.8555 (dd, $J = 8.1$ and 4.4 Hz, H-2), 4.8992 (t, $J = 5.7$ Hz, OH), and 8.472 (d, $J = 3.5$ Hz, NH); ^{13}C 59.0 (C-2), 60.5 (C-3) and 169.1 (C-1); **alanine** unit, ^1H 1.1285 (d, $J = 6.4$ Hz, H-3), 4.45 (m, H-2), and 7.3223 (d, $J = 7.5$ Hz, NH); ^{13}C 16.1 (C-3), 47.5 (C-2) and 172.5 (C-1); **isoleucine** unit, ^1H 0.7–0.8 (m, C(3)-CH_3, H-5), 0.74 (m, H-4), 1.52 (m, H-4), 1.7 (m, H-3), 4.017 (dd, $J = 8.6$ and 4.8 Hz, H-2) and 8.663 (d, $J = 4.5$ Hz, NH); ^{13}C 10.7 (C-5), 14.5 (C(3)-CH_3), 20.9 (C-4), 35.4 (C-3), 56.8 (C-2) and 170.3 (C-1); **proline** unit, ^1H 1.5 (m, H-4), 1.5 (m, H-3), 2.12 (m, H-3), 2.5 (m, H-5), 3.2 (m, H-5), and 4.412 (m, H-2); ^{13}C 29.9 (C-3), 40.1 (C-4), 45.3 (C-5), 60.3 (C-2) and 169.8 (C-1); **phenylalanine** unit, ^1H 3.14 (m, H-3), 4.1904–4.1122 (m, H-2), 7.133 (d, $J = 6.8$ Hz, H-ortho), 7.2202 (d, $J = 7.0$ Hz, H-para), 7.2844 (dd, $J = 6.8$ and 7.4 Hz, H-meta), and 8.5962 (d, $J = 7.9$ Hz, NH); ^{13}C 37.0 (C-3), 57.7 (C-2), 126.5 (C-para), 128.4 (C-meta), 128.6 (C-ortho), 137.6 (C-4) and 170.9 (C-1); **leucine** unit, ^1H 0.7–0.8 (m, H-5), 1.2567 (m, H-4), 1.52 (m, H-3), 4.28 (m, H-2), and 8.0455 (d, $J = 9.9$ Hz, NH); ^{13}C 21.2 and 23.1 (C-5), 24.3 (C-4), 14.6 (C-3), 51.5 (C-2) and 171.4 (C-1); **asparagine** unit, ^1H 3.05 (m, H-3), 4.3 (m, H-2), 7.2844 (brs, 1 H, C(4)-NH_2) 7.753 (d, $J = 5.5$ Hz (C-2)-NH) and 7.784 (brs, 1 H, (C-4)-NH_2); ^{13}C 35.9 (C-3), 49.6 (C-2), 171.6 (C-1) and 172.5 (C-4). Where the multiplicity is indicated (m) in the ^1H spectrum signal overlapping occurred.

Stylostatin 1 Chiral Assignments. The cycloheptapeptide (1 mg) was hydrolyzed with 1:1 propionic acid–hydrochloric acid (12 N) at 160 °C for 15 min.[8a] The corresponding amino acids were converted to *N*-pentafluoropropionyl isopropyl ester derivatives, and the configuration of isoleucine (retention time 16.75 m, standard L-Ile 16.83 and D-Ile 16.04 m) was established by chiral capillary chromatography.[8h]

Stylostatin 1 X-ray Structure Determination. A colorless crystal (0.18 × 0.46 × 0.04 mm) of stylostatin 1 from 2-propanol–water was used in the data collection. Crystal data: $C_{36}H_{54}N_8O_9 \cdot 2H_2O \cdot CH_3CHOHCH_3$, monoclinic space group $P2_1$, with $a = 9.056$ (2) Å, $b = 16.244$ (2) Å, $c = 14.585$ (4) Å, $\beta = 90.435$ (21)°, $V = 2145.5$ Å^3, λ (Cu Kα) = 1.541 84 Å, ρ_o (variable) = 1.237 g cm^{-3}, $\rho_c = 1.266$ g cm^{-3} for $Z = 2$ and FW = 839.0, $F(000) = 904$. All reflections corresponding to a complete quadrant, with $2\theta \leq 140°$ were measured at −104 °C using the $\omega/2\theta$ scan technique. Whenever possible, Friedel pair reflections were also measured immediately following each reflection. After Lorentz and polarization corrections, merging of equivalent reflections, and rejection of systematic absences, 7682 unique reflections remained. Of these, 7393 were considered observed ($I_o > 3\sigma(I_o)$) and were used in the subsequent structure determination and refinement. Linear and anisotropic decay corrections were applied to the intensity data as well as an empirical absorbtion correction (based on a series of psi-scans).[11] Near the conclusion of the refinement process, an additional least-squares absorbtion correction[12] was made in the data using the CRYSTALS[13] program DIFABS. Direct methods were utilized in the structure determination using SHELXS-86.[14] All 53 non-hydrogen atoms of stylostatin 1 were located on the first trial of SHELXS-86 with the value of TREF set at 400 and the remaining parameters at their default settings.

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Subsequent difference Fourier maps indicated the presence of additional areas of residual electron density which were assigned to two molecules of water and one partial molecule of 2-propanol. The remaining H atom coordinates were calculated at optimum positions for the cyclic peptide. The H atoms were added in the final stages of least-squares refinement and structure factor calculation processes, but were not refined. The cyclic peptide structure, in addition to the two solvate oxygen atoms from water and the three carbon atoms of 2-propanol, were refined in a full-matrix least-squares process with CRYSTALS, using the Robust-Resistant (Tukey and Prince) weighting method (Scheme 15 in CRYSTALS). Anomalous dispersion effects were included in F_c. The final cycle of refinement included 498 variable parameters (anisotropic refinement on all peptide non-hydrogen atoms and isotropic refinement on the five solvate atoms) and converged with unweighted and weighted standard crystallographic residuals of $R = 0.093$ and $Rw = 0.119$.

Final bond distances and angles were all within acceptable limits, except for the isopropyl group of leucine. During the whole refinement process, the C_{39}–C_{41} bond (1.41 Å) was consistently observed to be shorter than its supposedly, chemically equivalent neighbor, the C_{39}–C_{40} (1.58 Å). No logical explanation for this observation seems readily apparent.

The absolute stereochemical assignment for stylostatin 1 was based upon the known absolute stereochemistry determined (see above) for (*S*)-Ile. Thus, the absolute stereochemistry at the eight chiral centers of stylostatin 1 using the numbering shown in Figure 2[15] are as follows: 2*S*, 5*S*, 8*S*, 11*S*, 14*S*, 17*S*, 20*S*, and 45*S*. A final difference Fourier map showed some residual electron density in regions not associated with the main peptide molecule. These areas are believed to be due to additional, highly disordered solvate molecules. The higest peak in the final difference Fourier had a height of 1.13 e/Å^3. A computer-generated perspective drawing depicting the absolute configuration of stylostatin 1 is shown in Figure 2.

(15) Preparation of Figure 2 was accomplished with SHELXTL-PLUS: G. Sheldrick, Siemens Analytical X-ray Instruments, Inc., Madison WI 53719.

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Supplementary Material Available: A more detailed description of the X-ray data collection and refinement, X-ray crystallographic tables of atomic coordinates, bond lengths and angles, and anisotropic thermal parameters for stylostatin 1 (7 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

Herboxidiene: A Potent Phytotoxic Polyketide from *Streptomyces* sp. A7847

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Investigation of a secondary metabolite produced by *Streptomyces* sp. A7847 (ATCC 49982) has resulted in the identification of herboxidiene (1, $C_{25}H_{42}O_6$), a novel polyketide with exceptional phytotoxicity to several annual weed species. Synthetic modification studies have defined the permissible structural alterations of 1 which maintain biological activity. Significant structural change (see compounds 12 and 13) resulted in a loss of activity as did opening of the epoxide ring (see compounds 5–8) or oxidation of the secondary alcohol at carbon 18 (3).

Microorganisms have demonstrated a remarkable potential for producing secondary metabolites which are toxic to plants. These metabolites offer opportunities for controlling noxious weeds as products per se or by providing agricultural researchers with new structural models for analog synthesis.[2] In the continuation of a screening program to discover novel natural product herbicides from microbial sources,[3] *Streptomyces* sp. A7847 (ATCC 49982) was found to produce herboxidiene[4] (1), a novel polyketide which effectively and selectively controls several annual weed species at application rates as low as 7 g per acre. *Streptomyces* sp. A7847 was previously shown to produce the phytotoxic amino acid (2*R**,3*S**)-β-methyltryptophan.[5] This paper describes the isolation, structure determination, synthetic modification, and biological activity of herboxidiene and its derivatives.

Isolation and Characterization. Herboxidiene was produced in shake flask fermentations of *Streptomyces* sp. A7847. The sterile culture filtrate (1 L) was extracted with *n*-butanol, and the extract was purified by reversed-

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