Aureoverticillactam, a Novel 22-Atom Macrocyclic Lactam from the Marine Actinomycete *Streptomyces aureoverticillatus*†

Scott S. Mitchell, Benjamin Nicholson, Sy Teisan, Kin S. Lam, and Barbara C. M. Potts*

Nereus Pharmaceuticals, Inc., 10480 Wateridge Circle, San Diego, California 92121

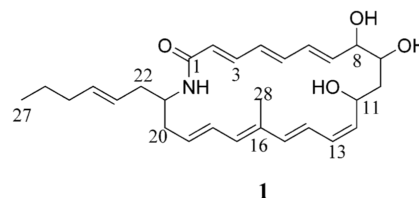
Received January 9, 2004

During the course of our screening program designed to discover novel anticancer and anti-infective agents from marine microorganisms, a strain of *Streptomyces aureoverticillatus* (NPS001583) isolated from a marine sediment was found to produce a novel macrocyclic lactam with cytotoxicity against various tumor cell lines. Using extensive MS, UV, and NMR spectral analyses, the structure has been established as compound **1**, aureoverticillactam, a 22-atom macrocyclic lactam incorporating both triene and tetraene conjugated olefins.

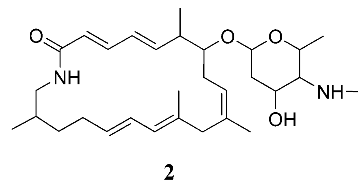
Marine-derived natural products are a rich source of potential new antibiotics and anticancer agents. As the late D. John Faulkner observed, "it now seems more obvious than ever that marine natural products should be regarded as the inspiration for new pharmaceuticals...."[1] The oceans are highly complex and house a diverse assemblage of microbes that occur in environments of extreme variations in pressure, salinity, and temperature. Marine microorganisms have developed unique metabolic and physiological capabilities that not only ensure survival in extreme habitats but also offer the potential to produce compounds that would not be observed from terrestrial microorganisms.[2,3] Therefore, efforts to culture novel microorganisms from the marine environment coupled with state-of-the art screening, chemistry, and structural elucidation capabilities should yield a significant source of novel metabolites for drug discovery.

Macrocyclic lactams comprise a small family of compounds produced by different *Streptomyces* species that have been shown to exhibit a wide variety of biological activities. Examples include the anticancer leads vicenistatin (**2**) and hitachimycin (**3**),[4,5] the antibiotics BE-14106 (GT-32A; **4**) and GT-32B (**5**),[6,7] and the endothelial adhesion inhibitor cyclamenol (**6**),[8] which represent 19- and 20-atom macrocyclic lactams. In this paper, we describe the isolation and structure elucidation of aureoverticillactam (**1**), which was produced by a strain of *Streptomyces aureoverticillatus*, family Streptomycetacea (Krasilnikov and Yuan, 1960),[9] isolated from a marine sediment. Compound **1** is the first example of a macrocyclic lactam with a 22-atom ring, therefore defining a novel carbon skeletal system. Although *S. aureoverticillatus* has not previously been reported to produce related macrocyclic lactam chemistry, it has been reported as a source of the streptorubin/prodiginine pigments,[10−12] and the Nereus marine isolate also produces a compound identified as either streptorubin A or B in yields higher than 2% dry extract weight.

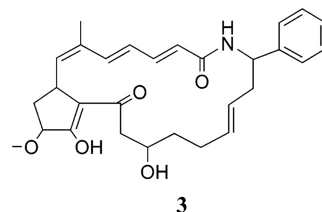
As part of our continued effort to exploit marine microbial natural products for drug discovery and development, an extract of the Nereus microbial strain NPS001583 was found to have cytotoxicity against HT-29 human colorectal adenocarcinoma cells at a concentration of 2.5 μg/mL. A bioassay-guided fractionation protocol utilizing C18 reversed-phase chromatography suggested that the growth inhibitory activity against HT-29 cells could be traced to both the known streptorubin and a second major metabolite later established to be compound **1**. Subsequent purification of **1** involved trituration of the crude extract with hexanes to remove the streptorubin and preparative scale C18 HPLC using an isocratic MeOH/H_2O gradient (75/25) to yield **1** in 95% purity and 1.6% isolated yield from dried crude extract.



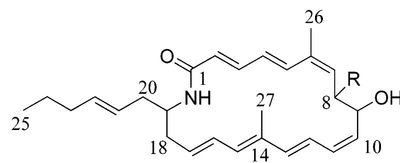
1



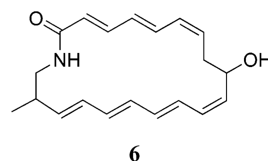
2



3



4: R = OH
5: R = H



6

† Dedicated to the late Dr. D. John Faulkner (Scripps) and the late Dr. Paul J. Scheuer (Hawaii) for their pioneering work on bioactive marine natural products.

* Corresponding author. Phone: 858-200-8324. Fax: 858-587-4088. E-mail: bpotts@nereuspharm.com.

10.1021/np049970g CCC: $27.50 © 2004 American Chemical Society and American Society of Pharmacognosy
Published on Web 07/03/2004

Table 1. ^{1}H and ^{13}C NMR Data for **4**[7] and **1** and HMBC Correlations for **1** in DMSO-d_6

	4			**1**		
pos	δ_C	δ_H	pos	δ_C	δ_H (mult, J (Hz))	HMBC
1	166.6		1	165.9		NH, H-2, H-3
2	123.8	5.90	2	125.5	5.83, 1H, d, 16.0	
3	139.5	6.65	3	138.0	6.74, 1H, dd, 15.1, 10.6	
4	124.9	6.14	4	129.8	6.21, 1H, m	H-2
5	142.5	6.14	5	137.4	6.33, 1H, m	H-3
6	131.5		6	129.4	6.27, 1H, m	
7	138.7	5.18	7	137.7	5.80, 1H, dd, 14.5, 5.3	H-5, OH(C8)
8	72.1	4.56	8	73.1	4.23, 1H, br m	H-6, H-7, OH(C8)
			9	73.8	3.80, 1H, m	
			10	38.2	1.46, 1H, m 1.29, 1H, m	
9	69.7	4.30	11	67.6	4.54, 1H, m	
10	131.3	5.40	12	136.3	5.39, 1H, m	
11	128.5	6.00	13	126.8	5.85, 1H, m	
12	123.8	5.90	14	122.8	6.15, 1H, m	H-15
13	136.3	6.08	15	137.3	6.12, 1H, d, 15.4	H-13, H-17, H-28
14	132.9		16	133.1		H-18, H-28
15	130.3	5.83	17	130.8	5.89, 1H, d, 12.3	H-18,[a] H-28
16	130.7	6.17	18	131.0	6.35, 1H, m	
17	129.9	5.42	19	130.5	5.49, 1H, m	H-18[a]
18	39.8	2.32 1.76	20	38.1	2.32, 1H, m 2.05, 1H, m	H-18
19	49.3	3.81	21	49.3	3.71, 1H, m	
20	38.0	2.14	22	38.3	2.19, 2H, m	H-23 or H-24
21	127.1	5.43	23	127.2	5.39, 1H, m	H-22, H-25
22	131.7	5.43	24	131.8	5.42, 1H, m	H-22, H-25, H-26
23	34.1	1.95	25	34.1	1.95, 2H, m	H-23 or H-24, H-26, H-27
24	22.0	1.32	26	22.1	1.31, 2H, m	H-25, H-27
25	13.4	0.84	27	13.4	0.85, 3H, t, 7.2	H-25, H-26
27	11.9	1.58	28	12.4	1.74, 3H, s	H-15, H-17
26	12.4	1.81				
NH	7.23				7.30, 1H, bd	
OH (C8)	4.84				4.67, 1H, br m	
OH (C9)	4.98				4.62, 1H, br m	
OH (C11)					4.51, 1H, br m	

[a] Interchangeable assignments.

A HRESI-TOF-MS measurement of M + H^{+} at m/z = 454.2962 (Δ_{calc} $C_{28}H_{40}NO_4$, 454.2957 = 1.1 ppm) was consistent with a proposed molecular formula of $C_{28}H_{39}NO_4$, requiring 10 double-bond equivalents. The UV spectrum (λ_{max}= 290 nm, shoulder 283, 310, 330 nm) compared favorably with that of macrocyclic lactams **4**, **5**, and **6**,[6−8] each of which comprise both a triene and a tetraene. The ^{13}C NMR and multiplicity-edited HSQC spectra of **1** indicated the presence of 28 carbons, including one carbonyl (δ_C 165.9), one quaternary olefinic carbon (δ_C 133.1, C-16), 15 olefinic methines (δ_C 122.8−138.0), three methines bearing oxygen (δ_C 67.6, 73.1, 73.8), one methine bearing nitrogen (δ_C 49.3), five aliphatic methylenes (δ_C 22.1−38.3), and two methyl groups (δ_C 12.4, 13.4). Additional support for these functionalities was evident in the ^{1}H spectrum of **1**, which included, among other signals, two methyl groups (δ 0.89 (3H, t, J = 7.2), H-27; 1.74 (3H, s), H-28), one amide proton (δ 7.30 (1H, bd), 3 hydroxy protons (δ 4.67 (1H, br m), OH (C8); 4.62 (1H, br m), OH (C9); 4.51 (1H, br m), OH (C11), and a series of heavily overlapped multiplets appropriate for the 15 conjugated olefinic protons in the proposed structure (Table 1). The one carbonyl signal and eight double bonds inferred from the 16 olefinic carbon signals accounted for nine double-bond equivalents, requiring the presence of one ring.

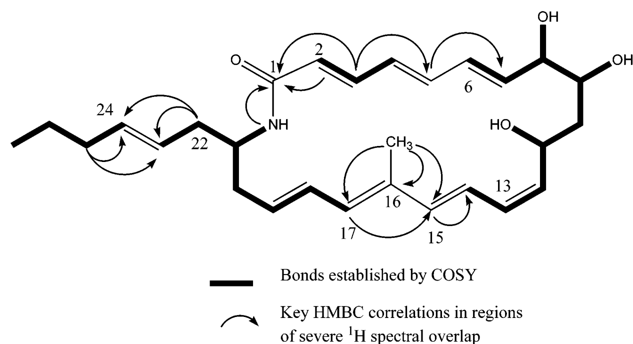


Figure 1. Key COSY and HMBC correlations establishing the structure of aureoverticillactam (**1**).

Connectivities for the functional groups of aureoverticillactam (**1**) were determined using a combination of ^{1}H COSY cross-peaks and ^{1}H−^{13}C HMBC correlations. Ideally, COSY correlations would have been sufficient to assign the structure of **1** using only two major proton spin systems. Unfortunately, extensive spectral overlap in the ^{1}H NMR spectrum did not allow use of COSY to establish connectivities for bonds C4−C5, C5−C6, C14−C15, and C23−C24. In fact, the latter two proton pairs suffered from nearly degenerate chemical shifts (Table 1). Nevertheless, the COSY effectively established four spin systems that were connected through two- and three-bond ^{1}H−^{13}C HMBC correlations (Figure 1). The two segments of the hexene side chain (H-22 to H-23 and H-24 to H-27) were connected through a series of reinforcing HMBC correlations (e.g., H-22 to C-24; H-25 to C-23), while the triene was established through HMBC correlations from H-3 to C-5 and H-5 to C-7. The tetraene was resolved through numerous HMBC correlations involving the branching methyl group (H-28 to C-15, C-16, and C-17), as well as correlations from H-15 to C-14 and H-17 to C-15, which bridged the C-16 quaternary carbon. Finally, the NH proton was correlated to the carbonyl carbon (C-1) in the HMBC spectrum, which was in turn linked to the H-2 to H-4 spin system via correlations from H-2 and H-3 to C-1, collectively defining the amide functional group and closing the lactam ring. Thus, a macrocyclic lactam containing 22 atoms was established.

Aureoverticillactam (**1**) is most closely related to compound **4**, but with the clear distinction of the insertion of two aliphatic carbons between the triene and tetraene systems in **1**. The NMR data for **1** and **4** compare favorably from C-14 through C-27 (atom numbering for **1**), with some exceptional proton resonances (e.g., H-14, H-18, and H-20$_b$; Table 1). In contrast, the ^{1}H and ^{13}C NMR values for the tetraene methyl substituent of **1** (C-28, δ_C 12.4; H$_3$-28, δ_H 1.74) are in better agreement with the values reported for the triene methyl substituent of **4** (δ_C 12.4, δ_H 1.81) than with those of the tetraene methyl substituent of **4** (δ_C 11.9, δ_H 1.58). Moreover, the structural differences between **1** and **4** noted above give rise to considerable ^{1}H and ^{13}C chemical shift differences in the range from C-4 to C-11.

In compound **1**, the geometry of the H-2/H-3 double bond was established as *trans* on the basis of the J values for the only completely resolved olefinic proton in the molecule, H-3 (J = 15.1, 10.6 Hz). The geometry of H-6/H-7 was similarly assigned as *trans* based on the coupling constants for H-7 (J = 14.5, 5.3 Hz). A selective irradiation 1D-TOCSY experiment (irradiation 4.53 ppm (H-11), 80 ms mixing time) allowed the direct measurement of couplings observed for H-12 (dd, 10.5, 7 Hz), supporting the assignment of the H-12/H-13 olefin in the *cis* geometry. Protons H-14 and H-15 give rise to an AB spin system for which

the upfield portion of the multiplet is partially resolved and reveals a large non-first-order coupling on the order of 15.1 Hz, suggesting a *trans* geometry. Assignment of the conformation of the remaining olefins was not possible due to spectral overlap, which precluded the measurement of $^{1}H-^{1}H$ proton coupling constants and/or NOEs. Aureoverticillactam (**1**) is soluble in DMSO at concentrations up to 20 uM (10 mg/mL) and sparingly soluble in other solvents such as water, methanol, acetone, and chloroform, circumventing efforts to improve the spectral resolution through solvent effects.

The growth inhibitory activity of aureoverticillactam (**1**) was evaluated in a series of assays targeting cancer cell lines. The EC_{50} values were 3.6 ± 2.6 μM against HT-29 cells, 2.2 ± 0.9 μM against B16-F10 cells, and 2.3 ± 1.1 μM against Jurkat cells, indicating that **1** has moderate activity against colorectal adenocarcnioma, melanoma, and leukemia cell lines, respectively.

Experimental Section

General Experimental Procedures. The reported UV spectral data were obtained from analytical HPLC analysis of the purified compound using an Agilent HP1100 HPLC equipped with an Agilent PDA detector; the mobile phase was a mixture of ACN and H_2O. NMR spectra were collected using a 500 MHz Bruker Avance spectrometer at 300 K in DMSO-d_6 using an inverse probe equipped with *x,y,z*-gradients, except for the ^{13}C NMR spectrum, which was acquired with a broadband observe probe. Mass spectra were acquired using a Micromass Q-Tof2 mass spectrometer with ES+ ionization. HRESI spectra were referenced using a poly(ethylene glycol) polymer mixture which was co-injected during acquisition as an internal accurate mass standard. All sequencing and analysis were completed at Accugenix, a division of Acculab Inc.

Biological Material. Production strain NPS001583 was identified as *S. aureoverticillatus* using sequence analyses of the 16S rRNA gene and subsequent matching of the resulting sequence against the validated MicroSeq database. Alignment of 500 base pairs of the 16S rRNA sequence resulting from strain NPS001583 differed by 0.70% from the MicroSeq data for *S. aureoverticillatus*, allowing for the species level taxonomic identification of strain NPS001583. The culture (strain NPS001583) was deposited on January 21, 2003, with the American Type Culture Collection (ATCC) in Rockville, MD, and assigned the ATCC patent deposition number PTA-4943.

Fermentation, Extraction, and Isolation of 1. Fresh seed culture for the production of NPS001583 compounds was prepared from frozen stock grown in vegetative medium consisting of the following per liter of seawater: starch, 10 g; yeast extract, 4 g; and peptone, 2 g. Five milliliters each of the seed culture was inoculated into the production medium consisting of the following per liter of seawater: starch, 5 g; Hydro Solubles, 4 mL; Menhaden fish meal, 2 g; kelp powder, 2 g; and chitosan, 2 g. The production culture (200 flasks) was incubated at 28 °C for 4 days on a rotary shaker operating at 250 rpm. Sterile XAD-16 resin (~3 g) was added to each flask. The flasks were returned to the shaker and incubated at 28 °C and 250 rpm for an additional 3 days. The culture broth was extracted with $MeOH/CH_2Cl_2$ (1:1, 20 L), and the extract was dried in vacuo.

The crude extract (1 g aliquots, 5 g total) was triturated with hexanes to remove nonpolar components, including streptorubin, and then dissolved in 100% DMSO for purification by C18 HPLC chromatography using a 75% methanol/25% water isocratic eluent. HPLC purification was carried out using an ACE 5 micron reversed-phase C18 column with column dimensions of 20 mm (i.d.) by 150 mm length for initial preparative separations and 10 mm by 250 mm for final purification. Compound **1** was isolated in 1.6% yield from dried crude extract in 95% purity (UV area % at 210 nm on analytical HPLC); alternatively, the isolated yield was calculated to be 2 mg/L of fermentation broth.

Aureoverticillactam (1): amorphous white solid; UV (acetonitrile/water) $\lambda_{max} = 290$ nm, shoulder 283, 310, 330 nm; ^{1}H NMR (DMSO-d_6), see Table 1; ^{13}C NMR (DMSO-d_6), see Table 1; HRESIMS *m/z* 454.2962 (calcd for $C_{28}H_{40}NO_4$, 454.2957).

Determination of in Vitro Cytotoxicity. The human cell lines HT-29 (colorectal adenocarcinoma) and Jurkat (acute T cell leukemia) and the murine cell line B16-F10 (melanoma) were obtained from ATCC. HT-29 cells were cultured in McCoy's 5A medium supplemented with 10% (v/v) FBS, 1% (v/v) penicillin/streptomycin, 2 mM L-glutamine, 10 mM HEPES, 1% (v/v) nonessential amino acids (NEAA), and 1 mM sodium pyruvate. Jurkat cells were cultured in RPMI 1640 with the same supplements as HT-29 cells. B16-F10 cells were cultured in DMEM supplemented with 10% (v/v) FBS, 1% (v/v) penicillin/streptomycin, 2 mM L-glutamine, and 10 mM HEPES. All cells were maintained in their respective media at 37 °C, 5% CO_2, and 95% humidified air. For the growth inhibition assays, cells were plated in 96-well plates at their appropriate densities. Adherent cells were plated the day before, and suspension cells were plated on the day of compound addition. Stock solutions of aureoverticillactam were prepared in DMSO. Serially diluted aureoverticillactam was added to the cells, resulting in a final concentration range of 20 μM to 2 pM. Forty-eight hours later, 0.2 mg/mL resazurin (Sigma) prepared in phosphate-buffered saline was added to each well and the cells were incubated for an additional 3−6 h. The fluorescence of the resazurin reduction product was measured using $\lambda_{ex} = 535$ nm and $\lambda_{em} = 590$ nm filters on a fluorimeter. The EC_{50} values were calculated in Prism (GraphPad Software Inc.).

Acknowledgment. We gratefully acknowledge the input and advice of our friends and collaborators Dr. W. Fenical and P. R. Jensen of the Scripps Institution of Oceanography. Help in the collection of the 1D-TOCSY data was generously provided by L. M. West.

Supporting Information Available: NMR spectra used to establish the structure of **1** are available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Faulkner, D. J. *Antonie van Leeuwenhoek* **2000**, *77*, 135−145.
(2) Fenical, W.; Sethna, K. M.; Lloyd, G. K. *Pharm. News* **2002**, *9*, 489−494.
(3) Okami, Y. *J. Mar. Biotechnol.* **1993**, *1*, 59−65.
(4) Shindo, K.; Kamishohara, M.; Odagawa, A.; Matsuoka, M.; Kawai, H. *J. Antibiot.* **1993**, *46*, 1076−1081.
(5) Umezawa, I.; Takeshima, H.; Komiyama, K.; Koh, Y.; Yamamoto, H.; Kawaguchi, M. *J. Antibiot.* **1981** *34*, 256−265.
(6) Kojiri, K.; Nakajima, S.; Suzuki, H.; Kondo, H.; Suda, H. *J. Antibiot.* **1992**, *45*, 868−874.
(7) Takahashi, I.; Ody, Y.; Nishiie, Y.; Ochiai, K.; Mizukami, T. *J. Antibiot.* **1997**, *50*, 186−188.
(8) Müller, H.; Bischoff, E.; Fiedler, V.; Weber, K.; Fugmann, B.; Rosen, B.; German Patent 4231289A1, 1994. US Patent 5,565,561, 1996.
(9) Krasilnikov, N. A.; Yuan, C. S. *Mikrobiologiya* **1960**, *29*, 482−489.
(10) Khokhlova, I.; Puchnina, A. V.; Artmonova, O. I. *Biokhimiia* **1964**, *29*, 841−845.
(11) Gerber, N. *J. Antibiot.* **1975**, *28*, 194−199.
(12) Wasserman, H.; Rodgers G.; Keith, D. *J. Am. Chem. Soc.* **1969**, *91*, 1263−1264.

NP049970G