# Lajollamycin, a Nitro-tetraene Spiro-β-lactone-γ-lactam Antibiotic from the Marine Actinomycete *Streptomyces nodosus*

Rama Rao Manam, Sy Teisan, Donald J. White, Benjamin Nicholson, Jennifer Grodberg, Saskia T. C. Neuteboom, Kin S. Lam, Deborah A. Mosca, G. Kenneth Lloyd, and Barbara C. M. Potts*

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

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A strain of *Streptomyces nodosus* (NPS007994) isolated from a marine sediment collected in Scripps Canyon, La Jolla, California, was found to produce lajollamycin (**1**), a nitro-tetraene spiro-β-lactone-γ-lactam antibiotic. The structure was established by complete analysis of spectroscopic data and comparison with known antibiotics oxazolomycin (**2**), 16-methyloxazolomycin (**3**), and triedimycin B (**4**). Lajollamycin (**1**) showed antimicrobial activity against both drug-sensitive and -resistant Gram-positive bacteria and inhibited the growth of B16-F10 tumor cells in vitro.

Marine actinomycetes are a rich source of new antibiotics and anticancer agents.[1] In an ongoing effort to discover drugs for the treatment of infectious diseases and cancer, we are continually mining ocean sediments for marine microorganisms that produce new chemistry with therapeutic potential. This effort led us to undertake a local expedition to the Scripps Canyon in La Jolla, California, a narrow gorge beginning just outside the breaker zone to the north of the pier at Scripps Institution of Oceanography and extending seaward in a southwesterly direction for about one mile, where it joins with La Jolla Canyon.[2,3] The topography of the sea floor in and around these submarine canyons is conducive to collecting marine sediments at a wide range of depths spanning a short distance, over which we conducted a transect, taking sediments at depths between 15 and 650 ft. From this collection, we isolated a strain of *Streptomyces nodosus* (NPS007994) that produced lajollamycin (**1**), a nitro-tetraene spiro-β-lactone-γ-lactam antibiotic, along with two new, unrelated secondary metabolites that will be described elsewhere.

The crude extract containing lajollamycin (**1**) was obtained by adding XAD-16 resin to the NPS007994 fermentation culture, filtering the culture broth to recover both the cell mass and XAD-16 resin, and extracting the cell mass/resin mixture with ethyl acetate. The extract was subjected to reversed-phase HPLC using an acetonitrile−water gradient, followed by a final HPLC purification step using a methanol−water gradient to obtain compound **1** in an isolated yield of 4 mg/L.

Lajollamycin (**1**) was obtained as a bright yellow solid. A HR ESI-TOF-MS measurement established the molecular formula as $C_{36}H_{53}N_3O_{10}$, requiring 12 degrees of unsaturation. The collective 1D (Table 1) and 2D NMR (Figure 1) data suggested the presence of several substructures common to the small family of triene spiro-β-lactone-γ-lactam antibiotics, including oxazolomycin (**2**),[4] 16-methyloxazolomycin[5] (**3**), and triedimycin B[6] (**4**). In fact, **1**, **3**, and **4** were identical in structure from C-1 to C-17, which includes the spiro-β-lactone-γ-lactam bicyclic ring system, across the amide linkage joining C-12 to C-1′, and through the C-1′ to C-9′ carbon chain, which contains the triene conjugated olefin. Interestingly, the NMR data for **1** indicated that the oxazole ring common to antibiotics **2**−**4** was absent. Moreover, the UV spectrum of **1** was distinctly

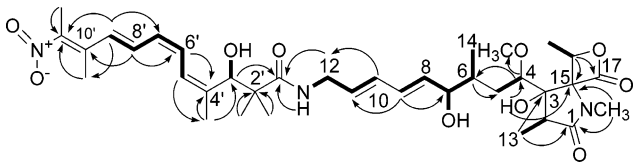
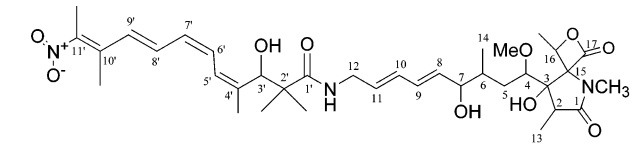
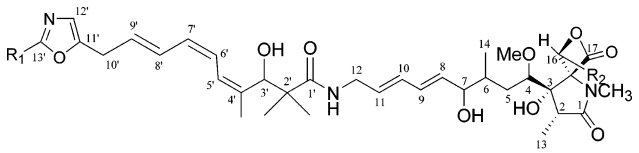


**Figure 1.** Key COSY (bold) and HMBC (arrow) correlations used to establish the structure of **1**.



**1**



**2** $R_1$=H, $R_2$=H
**3** $R_1$=H, $R_2$=$CH_3$
**4** $R_1$=$CH_3$, $R_2$=$CH_3$

different from those of **2**, **3**, and **4**, which reportedly do not absorb beyond 285 nm. Analysis of a series of NOESY (vide infra) and HMBC correlations (H-8′, H-9′, $H_3$-12′, and $H_3$-13′ to C-10′; H-9′ and $H_3$-12′ to C-11′) indicated that the triene system of **1** was extended by an additional double bond that was substituted with two methyl groups. The resulting substructure encompassed all of the required hydrogens and carbons, two of the nitrogens, and eight of the oxygens, leaving only $NO_2$ unaccounted for. The nitro group was therefore placed at the terminal carbon (C-11′, $\delta$ 146.0), giving rise to a nitro-tetraene conjugated olefin. While the novelty of this functional group precluded direct comparison of its UV spectrum with those of model compounds, reasonable estimates obtained by considering base values for substituted nitro-ethenes (e.g., 2-methyl-3-nitrobut-2-ene: $\lambda_{max}$ 262)[7] together with increments for extended conjugation support this structural assignment. Thus, the namesake trienyl-oxazole common to the triedimycins and oxazolomycin antibiotics was replaced by a unique nitro-tetraene in **1**.

The double-bond geometries of the tetraene system were established through analysis of proton−proton coupling

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

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**Table 1.** $^{13}C$ NMR Assignments for 16-Methyloxazolomycin (**3**)[5] in DMSO-$d_6$ and Triedimycin B (**4**)[6] in $CDCl_3$, and $^{13}C$ NMR and $^1H$ NMR Assignments for Lajollamycin (**1**) in $CDCl_3$

| | $\delta_C$ | | | $\delta_H$ (mult, $J$ (Hz)) |
|---|---|---|---|---|
| pos | **3** | **4** | **1** | **1** |
| 1 | 174.2 | 175.1 | 175.0 | |
| 2 | 44.7 | 43.1 | 43.0 | 2.43, 1H, q, 7.5 |
| 3 | 81.8 | 81.6 | 81.5 | |
| 4 | 83.7 | 82.0 | 82.0 | 3.67, 1H, t, 4.5 |
| 5 | 33.1 | 33.1 | 33.1 | 1.37, 1H, m |
| | | | | 2.02, 1H, m |
| 6 | 37.9 | 37.1 | 37.1 | 1.85, 1H, m |
| 7 | 76.4 | 77.0 | 76.9 | 3.94, 1H, br t, 6.5 |
| 8 | 129.5 | 134.2 | 134.2 | 5.67, 1H, m |
| 9 | 130.0 | 131.2 | 131.7[a] | 6.21, 1H, m |
| 10 | 131.5 | 131.6 | 131.1[a] | 6.16, 1H, m |
| 11 | 134.0 | 129.7 | 129.5[b] | 5.67, 1H, m |
| 12 | 42.2 | 41.2 | 41.2 | 3.90, 2H, m |
| 13 | 20.8 | 10.1 | 10.0 | 1.18, 3H, d, 7.5 |
| 14 | 17.2 | 17.6 | 17.7 | 0.96, 3H, d, 6.5 |
| 15 | 84.7 | 84.3 | 84.3 | |
| 16 | 78.4 | 77.5 | 77.5 | 4.84, 1H, q, 7.0 |
| 16-Me | 17.9 | NR[c] | 16.8 | 1.78, 3H, d, 7.0 |
| 17 | 170.2 | 169.7 | 169.6 | |
| 1′ | 176.2 | 178.1 | 177.9 | |
| 2′ | 46.7 | 44.7 | 44.7 | |
| 2′-$Me_a$ | 22.6 | 21.6 | 21.5 | 1.09, 3H, s |
| 2′-$Me_b$ | 26.1 | 26.1 | 26.0 | 1.33, 3H, s |
| 3′ | 74.4 | 75.7 | 75.6 | 4.64, 1H, d, 6.0 |
| 4′ | 141.0 | 138.4 | 141.2 | |
| 4′-Me | 20.9 | 19.5 | 19.8 | 1.83, 3H, br s |
| 5′ | 124.6 | 124.8 | 124.6 | 6.49, 1H, d, 12.0 |
| 6′ | 125.5 | 123.7 | 127.5 | 6.38, 1H, m |
| 7′ | 128.2 | 128.0 | 128.2 | 6.07, 1H, t, 11.5 |
| 8′ | 129.1 | 128.1 | 131.8[a] | 7.08, 1H, dd, 11.5, 15 |
| 9′ | 129.8 | 129.1 | 129.6[b] | 6.41, 1H, d, 15 |
| 10′ | 29.4 | 29.2 | 133.3 | |
| 10′-Me | | | 15.2 | 2.06, 3H, br s |
| 11′ | 151.1 | 150.1 | 146.0 | |
| 11′-Me | | | 15.8 | 2.29, 3H, br s |
| 12′ | 122.9 | 122.6 | | |
| 13′ | 152.1 | 160.8 | | |
| 13′-Me | | 13.9 | | |
| N-Me | 27.1 | 26.6 | 26.6 | 2.89, 3H, s |
| 4-OMe | 57.1 | 57.3 | 57.3 | 3.40, 3H, s |
| NH | | | | 6.36, 1H, m |
| 3-OH | | | | 3.18, 1H, s |
| 3′-OH | | | | 4.52, d, 6.0 |
| 7-OH | | | | 2.36, br d, 2.5 |

[a] Assignments interchangeable. [b] Assignments interchangeable. [c] NR = not reported.

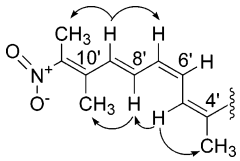


**Figure 2.** Key NOESY correlations used to establish the double-bond geometries of **1**.

constants, NOEDS, and NOESY data (Figure 2). The *E* geometry of the C-8′/C-9′ olefin was supported by large vicinal proton−proton coupling constants for H-8′ and H-9′ ($J$ = 15 Hz). NOESY correlations from H-8′ to $H_3$-10′-Me and from H-9′ to $H_3$-11′-Me in turn established the *E* geometry of the C-10′/C-11′ olefin. Independent confirmation of these NOEs was obtained through NOEDS experiments, in which irradiation at $H_3$-10′-Me gave rise to enhancement of the H-8′ multiplet, while irradiation at $H_3$-11′-Me resulted in enhancement of the H-9′ doublet. H-9′ was further correlated to H-7′ in the NOESY spectrum, while H-5′ was correlated to H-8′. These data, together with the H-6′/H-7′ coupling constant ($J$ = 11.5 Hz), established the *Z* geometry of the C-6′/C-7′ olefin. Finally, H-5′ was correlated to $H_3$-4′-Me in the NOESY spectrum, consistent with *Z*-geometry for the C-4′/C-5′ olefin. Thus, the double bonds of the conjugated triene system from C-4′ to C-9′ of **1** were of identical geometry (*Z, Z, E*) to those of **2**, **3**, and **4** and further extended by one *E* (C-10′/C-11′) olefin.

With respect to the diene system, $^1H-^1H$ coupling constants could not be delineated from the 1D $^1H$ NMR spectrum due to the complete or near degeneracy of the H-8/H-11 ($\delta$ 5.67, m) and H-9/H-10 ($\delta$ 6.21, m; 6.16, m) proton pairs. This chemical shift overlap also precluded assignment of the double-bond geometries from the NOESY spectrum. Furthermore, acquisition of the NMR data in DMSO-$d_6$ did not remove the problematic degeneracy. Despite similar reports of chemical shift overlap for the diene resonances of **3** and **4**, coupling constants consistent with *E* double-bond geometries were noted.[5,6] While the chemical shifts of the diene carbon and proton resonances of **1** and **4** are very similar, we sought independent evidence for the double-bond geometry as follows. Since the resonance frequencies were similar for the H-7 and $H_2$-12 protons flanking the diene, it was possible to simultaneously irradiate them ($\delta$ 3.91) in a 1D homonuclear decoupling experiment, allowing the residual H-8/H-9 and H-10/H-11 couplings to be observed. The resulting multiplet comprised a doublet ($J$ = 14.3 Hz) and a doublet of doublets ($J$ = 14.7, 4.0 Hz), consistent with *E,E* geometry.

The stereochemistry of the spiro-$\beta$-lactone-$\gamma$-lactam bicyclic ring system of oxazolomycin (**2**) was originally reported on the basis of X-ray crystallographic analysis of the *p*-bromobenzoate derivative of an ozonolysis product.[4] The stereochemistry of the bicyclic ring system of **3** was subsequently reported to be identical to that of **2**, and the additional chiral center at C-16 was assigned as *S* on the basis of observation of an NOE between $H_3$-16-Me and N-Me.[5] Interestingly, no such correlation was observed in the NOESY spectrum of **1**; instead, the methine proton, H-16, was correlated to N-Me. This observation was confirmed through a 1D NOEDS experiment, in which irradiation of N-Me resulted in enhancement of H-16. Thus, the relative stereochemistry of **1** and **3** appears to be different with respect to C-15 and C-16; in contrast, NOESY correlations between H-2 and H-4 suggest that the relative stereochemistries of C-2 and C-4 are the same.

Nitro groups are important yet relatively uncommon functional groups in natural products. According to AntiBase 2003 (John Wiley & Sons, Inc.), this functionality has been incorporated into approximately 150 reported microbial natural products or derivatives, representing less than 0.5% of reported microbial metabolites. The biosynthetic origin of nitro groups has generally been traced to oxidation of amine precursors,[8−10] although direct biochemical nitration has also been demonstrated.[11] In most natural products, the nitro group appears as a substituent of an aromatic or heteroaromatic ring, with representative compounds including pyrrolnitrin[12] and the pyrrolomycins,[13,14] and perhaps most notably the 2-amino-1-(4-nitrophenyl)-1,3-propane diols, including chloramphenicol.[15] Nitro groups are also represented within the carbohydrate units of some oligosaccharide antibiotics, such as the everninomycins.[16,17] To our knowledge, lajollamycin (**1**) is the first reported example of an acyclic nitro-tetraene conjugated olefin.

Efforts to obtain **1** in high purity (final HPLC purity: 93% (ELSD); 79% (UV area % at 210 nm)) were unsuccessful due to apparent degradation to related products that eluted at retention times similar to the parent

**Table 2.** MIC Values for Compound **1** against Bacterial Strains

| organism | MIC ($\mu$g/mL) |
|---|---|
| *Staphylococcus aureus* − methicillin sensitive | 4 |
| *Staphylococcus aureus* − methicillin resistant | 5 |
| *Streptococcus pneumoniae* − penicillin sensitive | 2 |
| *Streptococcus pneumoniae* − penicillin resistant | 1.5 |
| *Enterococcus faecalis* − vancomycin sensitive | 14 |
| *Enterococcus faecium* − vancomycin resistant | 20 |
| *Escherichia coli* − *imp* | 12 |

compound. We have monitored the stability of **1** by analytical HPLC and demonstrated that lajollamycin is light-sensitive. A DMSO solution of **1** (50 $\mu$M) degraded rapidly at room temperature when exposed to light, with only 25% of the parent compound remaining after 1 h. In contrast, **1** was stable in DMSO for at least 32 h when protected from light. Thus, appropriate precautions were taken to protect the compound from light during bioassays. Under these conditions, lajollamycin (**1**) showed antimicrobial activity against both drug-sensitive and -resistant microorganisms (Table 2) and inhibited the growth of murine melanoma cell line B16-F10, with an $EC_{50}$ of 9.6 $\mu$M.

## Experimental Section

**General Experimental Procedures.** NMR spectra were collected using a 500 MHz Bruker Avance spectrometer at 298 K in $CDCl_3$ using an inverse probe equipped with *x*,*y*,*z*-gradients, except for the $^{13}C$ NMR spectrum, which was acquired with a broad-band observe probe. The sample concentration was 20 mg/mL $CDCl_3$, and $^1H$ and $^{13}C$ chemical shifts were referenced to the solvent resonance at $\delta_H$ 7.24 and $\delta_C$ 77.0, respectively. The pulse programs used for NOEDS and homonuclear decoupling experiments were Bruker sequences noemul (1D sequence with presaturation) and zghd.2 (1D sequence with homodecoupling), respectively. 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. The reported UV spectral data were recorded on a Beckman-Coulter DU 640 spectrophotometer; the mobile phase was a mixture of ACN and $H_2O$.

**Biological Material.** Strain NPS007994 was isolated from a marine sediment sample collected at Scripps Canyons, La Jolla, CA, in 2001. Close to full length 16S rRNA sequence analysis of strain NPS007994 indicated that it is a strain of *Streptomyces nodosus*. The culture was deposited on January 7, 2004, with the American Type Culture Collection (ATCC) in Rockville, MD, and assigned the ATCC patent deposition number PTA-5747.

**Fermentation, Extraction, and Isolation of 1.** Fresh seed culture for the production of lajollamycin was prepared from frozen stock grown in the vegetative medium consisting of the following per liter of seawater: starch, 10 g; yeast extract, 4 g; and peptone, 2 g. Five milliliters of the seed culture was inoculated into 100 mL of production medium consisting of the following per liter of seawater: starch, 5 g; Hydro Solubles, 4 mL; fish meal, 2 g; kelp powder, 2 g; and chitosan, 2 g. The production culture (100 flasks) was incubated at 28 °C for 4 days on a rotary shaker operating at 250 rpm. Sterile XAD-16 resin (2−3 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 filtered through cheesecloth to recover the cell mass and XAD-16 resin. The cell mass and resin were extracted with 10 L of ethyl acetate. The extract was dried in vacuo. The dried extract (3.6 g), containing lajollamycin, was then processed for the recovery of lajollamycin.

The crude extract was chromatographed by reversed-phase HPLC on an ACE 5 C18-HL column (15 cm × 21 mm i.d.) by injecting 50 mg in each run at a flow rate of 14.5 mL/min using a solvent gradient of 100% $H_2O$ to 100% acetonitrile over 25 min. Fractions enriched in **1** were further purified using semipreparative HPLC using a ACE 5 C18-HL (10 mm × 250 mm i.d.) at a flow rate of 3 mL/min with a solvent gradient of 40% MeOH, 60% $H_2O$ to 100% MeOH over 30 min to obtain **1** (40 mg) as a bright yellow solid in an isolated yield of 4 mg/L.

**Lajollamycin (1):** bright yellow solid; mp 92−95 °C; $[\alpha]^{25}_D$ +75° (*c* 2 × $10^{-5}$, MeOH); UV (MeOH)) $\lambda_{max}$ ($\epsilon$) 229 (63 000), 306 (40 000), and 375 (22 000) nm; IR (AgCl) $\nu_{max}$ 3361 (br), 1820, 1683, 1643, and 1516 $cm^{-1}$; $^1H$ NMR and $^{13}C$ NMR ($CDCl_3$), see Table 1; HRESIMS *m/z* 688.3814 $(M + H)^+$ (calcd for $C_{36}H_{54}N_3O_{10}$, 688.3809).

**Sample Handling for Biological Assays.** Due to the light sensitivity of **1**, the compound was handled carefully in the dark. Plates were covered with aluminum foil to avoid light contamination, for example, while depositing compound, inoculating bacteria, and determining MICs. All of the assays were repeated in order to confirm the results, and similar purity results were observed when compound **1** was analyzed before and after bioassay time of test.

**Determination of Growth Inhibition of Murine Melanoma B16-F10 Cells**. The murine melanoma cell line B16-F10 was obtained from ATCC and was cultured in Dulbecco's modification of Eagle's medium supplemented with 10% (v/v) fetal bovine serum, 2 mM glutamine, 10 mM HEPES, and 1% (v/v) penicillin/streptomycin. The cells were cultured at 37 °C with 5% $CO_2$ and 95% humidified air. For the growth inhibition assays, B16-F10 cells were plated in 96-well plates the day before compound addition. Stock solutions of **1** were prepared in DMSO. Serially diluted **1** was added to the cells, resulting in final concentrations ranging from 20 $\mu$M to 2 pM. Forty-eight hours later, 0.2 mg/mL Resazurin (Sigma-Aldrich Chemical Co.) 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 fluorometer. The $EC_{50}$ values (the drug concentration at which 50% of the maximal observed growth inhibition is observed) were determined using a standard sigmoidal dose−response curve-fitting algorithm (XLfit 3.0, ID Business Solutions Ltd).

**Antibiotic Activity Assay.** The minimum inhibitory concentration (MIC) of lajollamycin was obtained against a variety of bacteria (Table 2) using a conventional broth dilution assay in accordance with standards recommended by the National Committee for Clinical Laboratory Standards (NCCLS).[18] The serial micro broth dilution method used BBL Mueller-Hinton broth (cation adjusted) except for the *Streptomyces pneumoniae*, which was tested in BBL Mueller-Hinton broth II (cation adjusted) with lysed horse blood. *S. pneumoniae* was incubated in a $CO_2$ incubator, whereas the others were not (standard NCCLS). The final bacterial inoculum contained approximately 5 × $10^5$ CFU/mL and was run on microtiter plates. The volume of each well was 100 $\mu$L, and the plates were incubated at 35 °C for 14−16 h. The MIC was defined as the lowest drug concentration that prevented visible growth of the bacterium.

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

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