Structure of the Spiroketal-macrolide Ossamycin

HERBERT A. KIRST,*,† JON S. MYNDERSE,†† JAMES W. MARTIN,†† PATRICK J. BAKER,††
JONATHAN W. PASCHAL,†† JORGE L. RIOS STEINER,††† EMIL LOBKOVSKY†††
and JON CLARDY*,†††

†Lilly Research Laboratories,
P.O. Box 708, 2001 West Main Street, Greenfield, Indiana 46140, U.S.A.
††Lilly Research Laboratories, Lilly Corporate Center,
Indianapolis, Indiana 46285, U.S.A.
†††Baker Laboratory, Department of Chemistry, Cornell University,
Ithaca, New York 14853-1301, U.S.A.

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Ossamycin is a cytotoxic agent of undetermined structure that was originally isolated in 1965 from culture broths of *Streptomyces hygroscopicus* var. *ossamyceticus*. Its overall structure and relative stereochemistry have now been determined by single crystal X-ray diffraction studies. Absolute stereochemistry was established according to the previously determined configuration of its aminosaccharide constituent, ossamine. The aglycone of ossamycin possesses a 24-membered macrolide ring system onto which is incorporated both a 6,6-spiroketal and a 5-membered hemiketal ring system. The overall three-dimensional structure possesses features in common with the related macrocyclic antibiotics dunaimycin, cytovaricin, and A82548A.

Ossamycin is a fermentation-derived natural product that was originally reported in 1965 as a novel cytotoxic agent isolated from culture broths of *Streptomyces hygroscopicus* var. *ossamyceticus*.[1] However, its structure has remained unknown until now. Preliminary studies in 1969 had shown that ossamycin contained an unusual aminodeoxysaccharide that was given the name, ossamine.[2] This amino sugar and its enantiomer have been prepared by several total syntheses and are well characterized.[2~5]

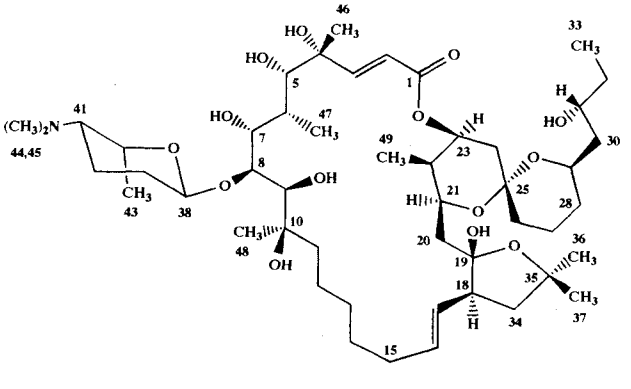
Aminodeoxy sugars such as ossamine are typically found in macrolides and other polyketide-derived fermentation products.[6] Ossamine itself has been recently proposed as a constituent of the novel tetracyclic insecticidal macrolide, spinosyn G (factor G of the A83543 complex).[7] As a result of our continuing interest in this large and structurally diverse class of polyketide-derived natural products, we have now determined the structure of ossamycin by single crystal diffraction X-ray crystallography (Fig. 1).

Isolation and Characterization

The sample of ossamycin was obtained by fermentation of its producing organism, *Streptomyces hygroscopicus* var. *ossamyceticus*, that was obtained from the American Type Culture Collection (Rockville, Md.) where it had been originally deposited by the Bristol-Myers Company under accession number 15420. Ossamycin was isolated from the mycelial mass by extraction with acetonitrile, separation using an HP-20ss non-functionalized resin, and then chromatography, first on silica gel and then by reversed-phase HPLC. The isolated sample was fully characterized by physico-chemical and spectroscopic methods and shown to be identical to a sample that had been previously received from the Bristol Laboratories by HPLC (including comparison by co-injection), mass spectrometry, and NMR (in acetone-d_6) spectroscopy.

After the structure of ossamycin had been established by X-ray crystallography, ^{1}H and ^{13}C NMR assignments were made on the basis of 1D comparisons with the corresponding spectra of cytovaricin and A82548A combined with DEPT, COSY, TOCSY, HMQC, HMBC,

Fig. 1. Structure of ossamycin.



and HMQC-TOCSY experiments (see Table 1).[8,9] The NMR spectra were collected in acetone-d_6 because the compound appeared to be stable in this solvent. In contrast, spectra collected in $CDCl_3$ developed a second component over time that appeared to result from opening the five-membered hemiketal ring, a conclusion based on the appearance of a new resonance at 207.25 in the ^{13}C NMR spectrum that was attributed to a ketone carbonyl group.

Most of the protons were assigned using COSY and/or TOCSY techniques. The remaining protons and carbon atoms were assigned by HMBC (long range carbon/proton correlations) and HMQC-TOCSY experiments, which also confirmed the assignments that had been made (Table 1). H-8 and C-8 were assigned through the observation of long range correlations from H-38 to C-8 and from H-8 to C-38. C-7 and its attached proton were assigned through COSY correlation between H-7 and H-8 and HMBC correlations between H-8 and H-47 to C-7, an assignment that differs from that reported in the literature for cytovaricin, but is consistent with previous work in this laboratory.[8,9]

Table 1. The NMR data for ossamycin in acetone-d_6.

Position	^{13}C	^{1}H
1	164.98	—
2	119.52	6.09
3	151.12	6.99
4	74.96	—
5	80.07	3.83
6	36.49	1.78
7	78.05	3.97
8	84.32	3.89
9	75.34	3.04
10	75.50	—
11	40.89	1.96/1.18
12	24.45	1.48/1.26
13	30.91	1.41/1.06
14	30.11	1.50/1.38
15	33.34	2.30/1.95
16	134.20	5.42
17	130.29	5.46
18	54.72	2.54
19	106.85	—
20	39.37	2.11/1.48
21	68.75	4.35
22	35.45	2.15
23	70.06	5.24
24	36.16	1.79/1.77
25	98.92	—
26	34.77	1.76/1.53
27	19.88	1.75
28	31.68	1.56/1.28
29	66.50	4.19
30	45.20	1.56/1.40
31	68.53	3.89
32	30.66	1.45/1.40
33	10.46	0.95
34	44.00	2.00/1.78
35	81.81	—
36[a]	30.61	1.40
37[a]	29.00	1.21
38	98.73	5.00
39	30.75	1.99/1.46
40	20.85	1.86/1.62
41	63.08	2.28
42	72.78	4.44
43	13.58	1.24
44/45	43.50	2.20
46	28.78	1.33
47	5.99	0.92
48	22.55	1.12
49	6.59	0.83
19-OH	—	5.33

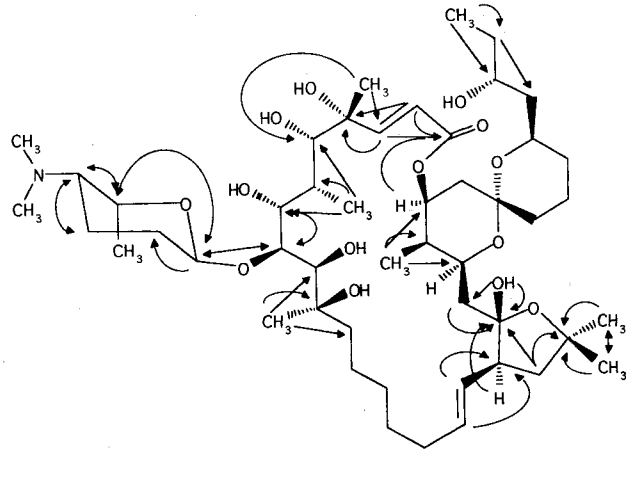
[a] Assignments may be interchanged.

C-19 and C-25 were distinguished by comparison with cytovaricin and through the HMBC correlations of 19-OH, H-20, H-34, and H-18 to C-19. The singlet methyl groups were assigned through HMBC correlations to their adjacent neighbors. C-36 and C-37 were not distinguished. Some of the observed HMBC correlations are shown in Fig. 2.

Crystallization and X-Ray Crystallography

Crystals suitable for X-ray analysis were grown from ethanol-water mixtures by vapor diffusion. There were two molecules (I and II) in the asymmetric unit of the crystal (space group $P2_1$, $Z=4$). The geometry of the two molecules (I and II) was approximately the same, and their macrocyclic moieties were virtually identical (r.m.s. = 0.16 Å), although high thermal motion and disordered regions made a detailed comparison difficult. In molecule I, the aminosugar has its ring oxygen atom, C-methyl group, and N-methyl groups disordered (see Fig. 3) and appears to have two conformations. There is another disordered region in molecule I at C-13 and C-14 (Fig. 3). Molecule II has only the side chain atoms at C-30 to C-32 disordered.

Fig. 2. HMBC correlations for ossamycin.



There are several intramolecular hydrogen bonds in ossamycin (O–O distances in the range of 2.58 ~ 2.74 Å), and four oxygen atoms form a "chain" of hydrogen bonds from the C-4 hydroxyl to the C-5 hydroxyl to the C-7 hydroxyl to the ring oxygen of ossamine. Another hydrogen bond is present from the C-19 glycosidic hydroxyl to the spiroketal oxygen atom between C-21 and C-25. There are also two short intermolecular O–O distances which indicate probable hydrogen bonds: from the C-9 hydroxyl in one molecule to the C-9 hydroxyl in a neighboring molecule (2.83 Å), and from the C-9 hydroxyl in one molecule to the C-10 hydroxyl in a neighboring molecule (2.80 Å).

Fig. 3. ORTEP drawing of ossamycin showing disorder within the crystal at two positions.

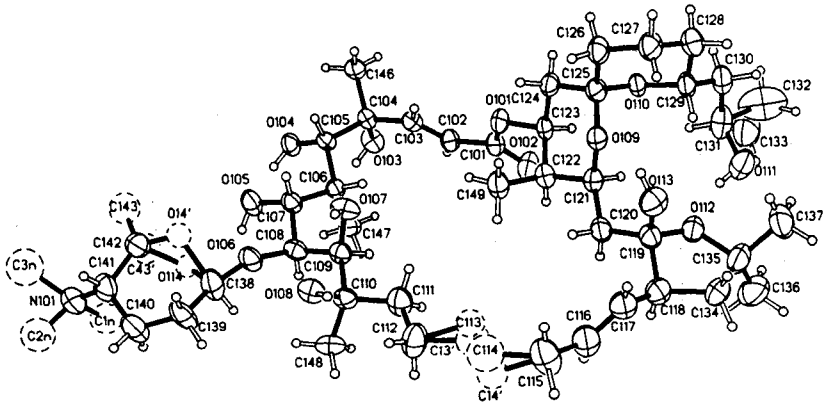


Fig. 4. Comparison of absolute stereochemistry of ossamycin, A82548A, and cytovaricin.

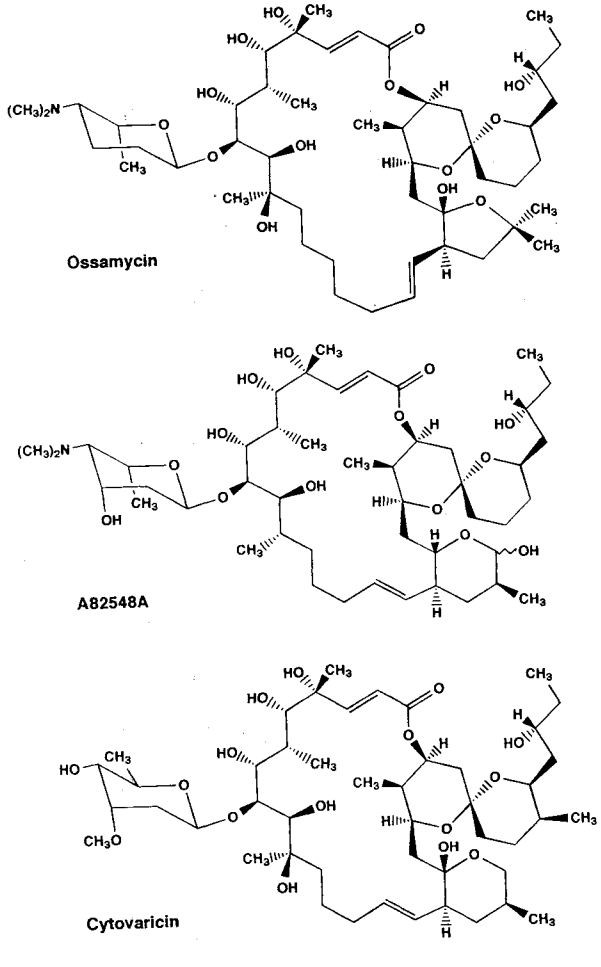
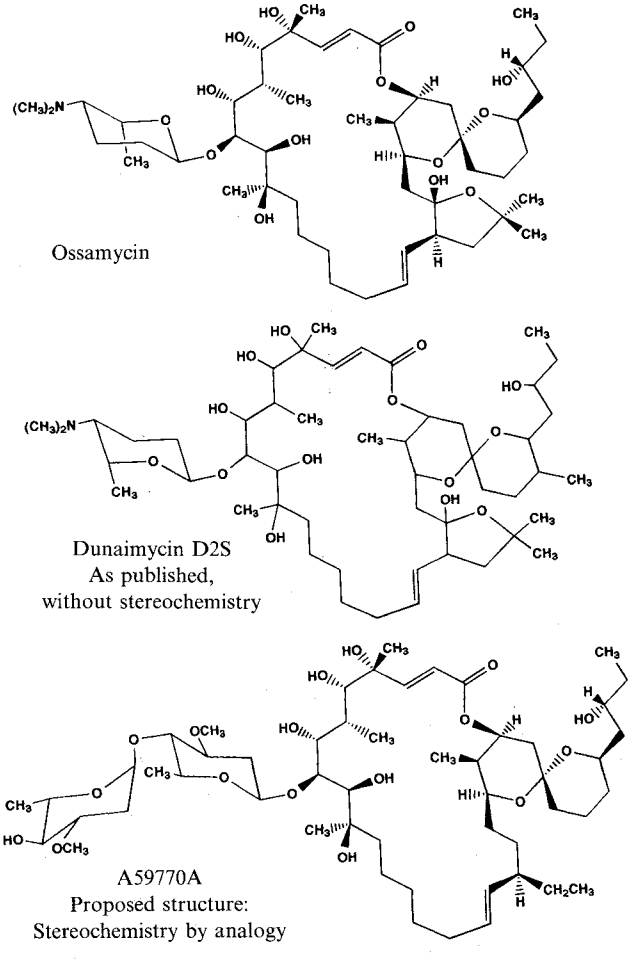


Fig. 5. Comparison of ossamycin, published structure of dunaimycin, and proposed structure of A59770A.



Discussion

Although ossamycin was discovered thirty years ago, its structure has remained unknown until the present time. This X-ray crystal study has now revealed that it is a new member of the family of spiroketal-containing macrolides in which several different types of hemiketal moieties are fused onto the larger macrocyclic and spiroketal ring systems (Fig. 4).[8~10] This interesting family of polyketide-derived fermentation products continues to increase in number as new members are isolated and identified. Ossamycin appears to be most closely related to the recently reported dunaimycin complex (Fig. 5), although most of the stereochemistry for the eight factors of dunaimycin was not reported in that original publication.[11]

The number of examples have been steadily increasing in which the relative and absolute stereochemistries have been analogous or identical within the aglycones of various members of the spiroketal-macrolide family (see Fig. 4). This trend now encompasses ring systems of 22-members (cytovaricin[8,12~14] and A82548A[9]), 24-members (ossamycin), and 26-members (oligomycin and rutamycin).[10,12] Therefore, it appears reasonable to anticipate that ossamycin and dunaimycin may also possess the same relative and absolute stereochemistry. If so, factor D2S of dunaimycin would then differ from ossamycin only by the presence of a methyl substituent at C-28 in the former's spiroketal ring system.

Other closely related compounds within this series include W719A and B,[15] antibiotics NK154183A and B,[16] and algacidin A and B.[17] Furthermore, based upon previous degradation studies, it is likely that another member of this family, A59770A, may also contain the same overall macrocyclic ring system as ossamycin, although it differs significantly in the C-18, C-19 region of the aglycone and in its saccharide substituents (Fig. 5).[18] The elucidation of structures for all of these complex, large-ring macrolides may lead to a better understanding of their differences in biological activities and should allow medicinal chemists to conduct more detailed analyses of structure-activity relationships and computer modeling of the structures within this family.

Experimental

General Methods

NMR data were collected on a Bruker AMX-500 NMR spectrometer using standard conditions for each experiment. Infrared spectra were measured on a Nicolet 510P optical bench spectrometer, ultraviolet spectra were obtained on a Shimadzu UV-2101 PC UV-VIS scanning spectrophotometer, and optical rotations were determined on a JASCO DIP-370 DIG-CP digital polarimeter. Electrospray mass spectra were obtained on a Perkin-Elmer Sciex API III spectrometer and FAB-MS data were obtained on a VG ZAB2-SE spectrometer. X-ray crystallographic data were collected with a Siemens R3m diffractometer using monochromated CuKα radiation ($\lambda = 1.5418$ Å).

Isolation of Ossamycin

Fermentation broth (222 liters) was filtered through a ceramic filter and the residual mycelium was washed with water (90 liters) and then extracted with acetonitrile (2 × 50 liters). The organic extract was diluted with water (300 liters) and loaded onto a column of Diaion HP-20ss (Mitsubishi, 10 liters). The effluent (400 liters) and water wash (30 liters) from the column were discarded and the crude product was eluted with acetone. Fractions containing ossamycin were combined and evaporated to dryness under reduced pressure. This procedure was repeated with another 222 liters of fermentation broth and the combined crude product was dissolved in dichloromethane (100 ml) and applied to a column of silica gel (EM Science, grade 62, 5.5 × 50 cm) set up in dichloromethane. After eluting with dichloromethane (3 liters), product was eluted with acetonitrile and the fractions containing ossamycin were combined and concentrated under reduced pressure. The residual oil was chromatographed by repeated preparative HPLC on a C18 column (25 cm × 4.14 cm i.d. Rainin Dynamax-60A, 8 μ C18 column equipped with a 5 cm × 4.14 cm i.d. guard module with the same packing), eluting with acetonitrile - water (7 : 3). Fractions were assayed by analytical HPLC on a C18 column (YMC-Pack ODS-AQ, 10 cm × 4.6 mm i.d., 5 μ spherical, 120 Å pore size), with a mobile phase consisting of a linear gradient of acetonitrile - water (7 : 3) to acetonitrile, and UV detection ($\lambda = 225$ nm); the retention time of ossamycin was 6.24 minutes. Fractions containing pure product were collected and those containing impurities were rechromatographed as before. The combined fractions of pure material were dissolved in dioxane (10 ml) and lyophilized to yield 1.7 g of ossamycin as a colorless solid: mp 186~188°C [lit.[1] 185~187°C]; $[\alpha]_D^{20^\circ} = +7^\circ$ (*c* 1, $CHCl_3$) [lit.[1] $[\alpha]_D^{23^\circ} = +8^\circ$ (*c* 1, $CHCl_3$)]; IR ν_{max} (KBr) 3474, 2940, 1720, 1456, 1382, 1272, 1229, 1168, 1121, 1085, 1055, 1004, 971, 910 cm^{-1}; UV (EtOH) λ_{max} nm ($\log \varepsilon$): shoulder at 215 (1.15) on end absorption; ^1H and ^{13}C NMR: see Table 1; positive ion electrospray MS *m/z* 912.7 $(M+H)^+$; electrospray MS/MS of 912.7 ion: *m/z* 894 $(M+H-H_2O)^+$ and 142 $(C_8H_{16}NO)^+$; high resolution FAB-MS *m/z*: Calcd. for $C_{49}H_{84}NO_{13}$ $(M+H-H_2O)^+$: 894.5943. Found: 894.5964.

X-ray Crystallographic Studies

Since the crystals diffracted poorly, an attempt was made to improve the resolution with low temperature. Unfortunately, all such attempts led to extremely broad diffraction maxima at temperatures below −20°C. Warming to room temperature restored the initial diffraction maxima.

A flat colorless crystalline plate of ossamycin measuring 0.06 × 0.26 × 0.38 mm^3 was investigated on a Siemens R3m diffractometer and showed monoclinic

symmetry. Unit cell dimensions of $a=11.957(4)$, $b=20.11(2)$, $c=22.517(7)$ Å, and $\beta=90.91(3)°$ were obtained from a least-squares fit of 25 well centered reflections with $35°<2\theta<45°$. The cell volume was 5413(5) Å^3, with a calculated density of 1.119 g/mm^3. The space group was $P2_1$, $Z=4$ (two molecules in an asymmetric unit). Intensities of 6137 diffraction maxima ($2\theta<100°$) were measured using $\theta/2\theta$-scans with a variable scan speed (2.0~29.3 degrees/minute), and 5774 were symmetry unique ($R_{int}=0.0286$). Three check reflections were measured every 97 reflections, and no significant variations in intensities were found. After correction for Lorentz and polarization effects, 4821 were judged observed ($F_o>2\sigma(F_o)$). This data set was called #1, and a second set was then collected.

For set #2, data were collected on the F2 station at CHESS using second crystal ($0.05\times0.20\times0.30$ mm) synchroton radiation and a Charged Couple Device detector, $\lambda=0.504$ Å, by the oscillation method.[19] Once again, the mosaicity spread was very high at low temperature, so the data were collected at room temperature. A total of 207° of data was collected in 3°90 s oscillations with a symmetric detector at 44 mm. Overall, 33,457 observations of 6,660 unique reflections were integrated to 1.0 Å resolution, with the average $R_{sym}=0.042$.

Numerous attempts to solve the structure using data sets #1 and #2 individually failed. Both data sets were scaled together using SHELXS, resulting in 7105 unique reflections, $R_{merg}=0.0526$, of which 5317 were judged observed. The structure was solved by direct methods (SHELX-86).[20] For the best solution, CFOM was 0.112 and NQUAL was −0.379. The E-map showed almost all of the non-hydrogen atoms. The R_E was 0.243 for 117 surviving atoms. The remaining atoms were located in subsequent difference Fourier syntheses. Full-matrix least-squares refinements on F^2 (SHELX-93) with anisotropic non-hydrogen atoms and isotropic riding hydrogen atoms converged to $R_1=7.0\%$, $wR_2=19.9\%$, GOF = 1.003. The multiplicity factor was set at 0.5 for partitions of atoms disordered into two positions; for the atoms disordered into three positions, the multiplicity was set at 1/3. A final difference Fourier map revealed no peaks greater than 0.41 e/Å^3.

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