Structure of the New Spiroketal-Macrolide A82548A

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(Received for publication April 25, 1995)

A new member of the spiroketal-containing macrolide class of fermentation-derived natural products was isolated from mycelial extracts of *Streptomyces diastatochromogenes*. The principal component, A82548A, was shown to possess a 22-membered macrolide ring system onto which was incorporated both a spiroketal and a hemiketal moiety. Relative stereochemistry was established by single crystal X-ray diffraction studies. Absolute stereochemistry was determined *via* hydrolysis of the amino sugar glycosidically linked to the aglycone, which was identified as L-kedarosamine. The overall three-dimensional structure is closely related to that of the macrolides cytovaricin, rutamycin, and ossamycin.

The collection and fermentation of soil-derived microorganisms and screening of their culture broths continue to provide an important source of novel natural products possessing potentially useful biological activities.[1~4] During the course of a screening program to discover new fermentation-derived compounds, antifungal activity was detected in the culture broth of A82548, an organism subsequently classified as a strain of *Streptomyces diastatochromogenes*. (Taxonomy was performed by Mr. F. P. MERTZ in the Lilly Research Laboratories, unpublished data) This report describes the complete structure elucidation of A82548A, the principal factor produced in this fermentation, and its structural interrelationships with the prototypic spiroketal-containing macrolide cytovaricin and other related members of this family.[5~8] The biological activities of these compounds will be reported separately.

Isolation and Characterization

The desired products were concentrated predominantly within the mycelium of the microorganism, with only about 10% of the total product present in the culture filtrate. Isolation of the active components in the A82548 complex was accomplished by extraction of the filtered biomass followed by chromatography on silica gel. Purification of the principal factor, designated as A82548A, was subsequently achieved by further chromatography on Sephadex LH-20 followed by crystallization from methanol-water.

A82548A was characterized by physicochemical and spectroscopic methods as a new polyketide-derived molecule whose gross structure (Fig. 1) was most closely related to cytovaricin, another complex spiroketal-containing macrolide that had previously been isolated from the culture broths of a different strain of *S. diastatochromogenes*.[5~8] More recently, structurally related macrolide aglycones lacking any saccharide substituents have been reported, such as phthoramycin, kaimonolide, and SS49, although no stereochemical details have been reported for these newer compounds.[9~11] The relative and absolute stereochemistry of cytovaricin has been further substantiated by its recent total synthesis and stereochemical correlations within the ring systems and substituents of several related macrolides have been described.[12,13]

Based upon mass spectral analyses and NMR spectroscopic comparisons with cytovaricin, the tentative structure depicted in Fig. 1 was proposed for A82548A. It clearly differed from cytovaricin by 1) lack of a tertiary hydroxyl group at C-10, 2) lack of a methyl group on the spiroketal ring system at C-26, 3) a different hemiketal ring system fused to the macrolactone at C-16 and C-17, and 4) an amino sugar rather than a neutral sugar attached to the C-8 hydroxyl group (Fig. 1). MS/MS data

† Retired.

Fig. 1. Gross structure of A82548A deduced from MS and NMR spectroscopic comparisons with cytovaricin.

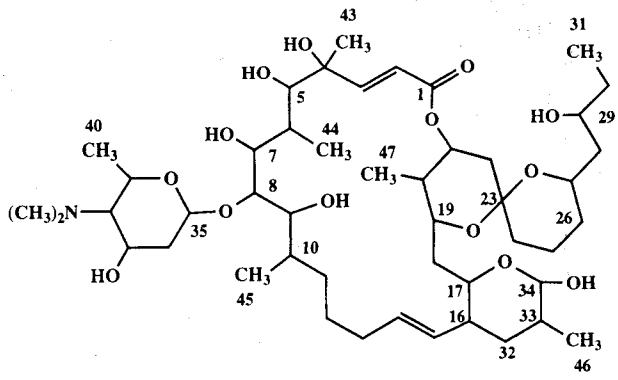


Table 1. The NMR assignments for A82548A in $CDCl_3$ solution.

Position	^{13}C	^{1}H
1	165.22	—
2	119.50	6.20
3	149.53	6.88
4	74.06	—
5	79.37	3.85
6	35.56	1.72
7	76.64	3.99
8	84.05	3.61
9	74.60	2.89
10	37.63	1.45
11	31.17	1.67/0.92
12	27.83	1.62/1.26
13	32.88	2.39/1.97
14	132.87	5.25
15	131.77	5.10
16	47.21	1.94
17	74.54	3.48
18	36.55	1.98/1.21
19	63.74	3.94
20	34.32	2.14
21	70.43	5.26
22	35.52	1.81/1.76
23	97.05	—
24	35.25	1.72/1.44
25	19.08	1.92/1.61
26	31.70	1.54/1.25
27	65.52	3.87
28	43.84	1.55/1.43
29	69.17	3.97
30	30.47	1.46
31	9.72	0.97
32	38.28	1.71/1.12
33	36.85	1.54
34	101.33	4.31
35	96.20	4.98
36	36.27	2.14/1.76
37	64.59	4.32
38	64.78	2.31
39	71.45	4.41
40	15.56	1.50
41/42	43.73	2.41
43	28.62	1.36
44	5.26	0.89
45	15.86	0.84
46	16.55	0.96
47	6.76	0.70

showed that fragments derived from the C-19 to C-31 portion of cytovaricin, SS49, and A82548A were very similar, indicating that a similar spiroketal ring system was present in each of them. MS/MS comparisons of several dimethylaminodeoxy sugars found in other polyketide-derived molecules (*e.g.*, spiramycin, ossamycin, erythromycin, angolamycin, tylosin) suggested that A82548A contained a 4-dimethylamino-2,4,6-trideoxy hexose. Although the NMR data (Table 1) suggested that the relative stereochemistry of A82548A was very similar to that of cytovaricin, it was not possible to unambiguously assign the stereochemistry at each of the asymmetric centers in the proposed structure.

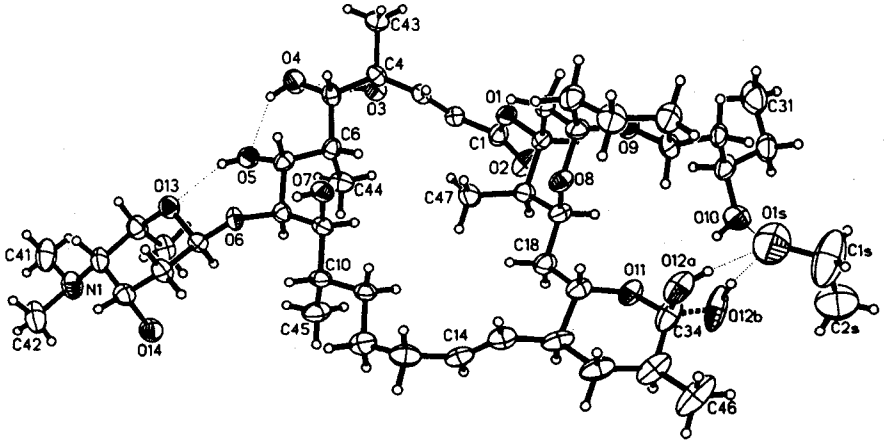
Crystallization and X-Ray Crystallography

Crystallization of the compound from ethanol-water yielded crystals in which two molecules of an A82548A-EtOH complex form the asymmetric unit. Interestingly, both anomers at C-34 are found in the asymmetric unit with an α/β ratio of 6 : 4 for each independent A82548A. Since these molecules can interconvert, this ratio probably reflects the thermodynamic equilibrium for a molecule rather than an artifact of crystal packing. An ORTEP drawing showing both possible anomeric structures at C-34 (O12a and O12b) is shown in Fig. 2.

The ring system of A82548A assumes an overall rectangular conformation, in which the spiroketal and sugar moieties project in opposite directions and the anomeric hemiketal forms one corner of the rectangle. A close interaction is observed between the substituents attached to the C-6 and C-9 methine units and the C-47 methyl group, which project toward the center of the molecule. A series of hydrogen bonds is present involving the hydroxyl groups of the C-4 to C-7 polypropionate segment of the macrolide. An additional hydrogen bond, which involves the ether oxygen atom of β-kedarosamine, fixes the saccharide's orientation. The hydroxyl groups at the anomeric center (C-34) and the extended side chain (C-29) are brought closer together within the same molecule *via* hydrogen bonding with a solvent molecule. The hydroxyl group at C-29, in conjunction with the hemiketal oxygen (O11), also interacts intermolecularly with the hydroxyl group at C-7 of the adjacent asymmetric unit, thereby building an extended ribbon of macrolide units throughout the crystal.

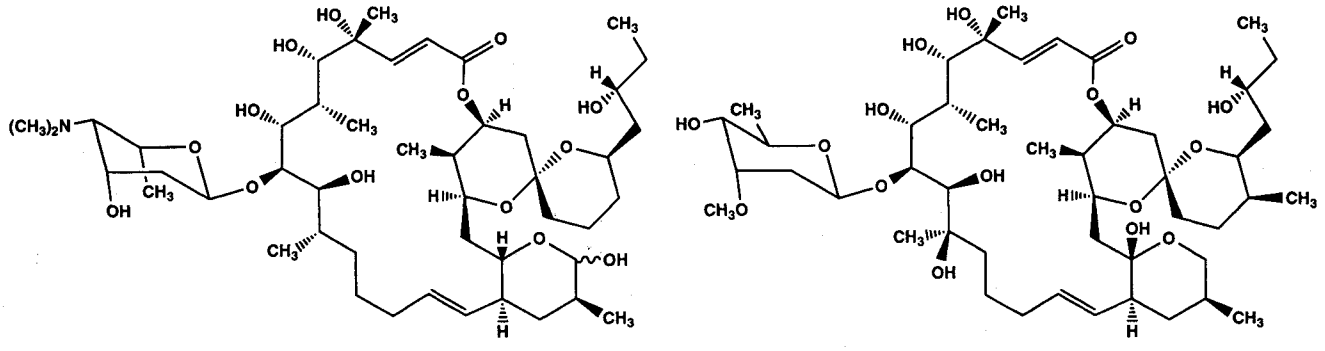
Both molecules in the asymmetric unit are essentially superimposable on each other. The average atomic displacement in the ring system corresponds to an r.m.s. of approximately 0.1 Å. The overall rectangular geometry along with the projection of the C-47 methyl group is

Fig. 2. A perspective view of A82548A as defined by X-ray diffraction. Heteroatoms (O or N) have crosshatches; carbons and hydrogens do not.



Hydrogen bonds are shown as dotted lines, and the thermal elipsoids for the nonhydrogen atoms are at the 50% level. Atom O12 is disordered over two orientations.

Fig. 3. Absolute stereochemistry of A82548A and cytovarcin.



A82548A Cytovaricin

reminiscent of other closely related antibiotics such as cytovaricin, rutamycin A,[12,13] and ossamycin.[14]

Absolute Stereochemistry

From examination of the X-ray structure, the saccharide was observed to possess the relative configuration of kedarosamine, an amino sugar recently identified in the structurally unrelated enediyne-containing fermentation product, kedarcidin.[15~17] The absolute configuration of this naturally occurring amino sugar was subsequently established as L-kedarosamine by the unambiguous synthesis of its methyl α-glycoside and the optical rotation of this saccharide was reported.[18] A very recent diastereoselective synthesis of methyl α-kedarosaminide has further confirmed the data assignments for this saccharide.[19]

Methanolysis of the amino sugar from A82548A under acidic conditions yielded a mixture of the methyl α- and β-glycosides in a 7:1 ratio as determined by NMR analysis of the product (Table 2). This ratio is similar to that reported for the analogous acidic methanolysis of kedarcidin, which produced a 9:1 ratio of the methyl α- and β-glycosides of kedarosamine.[15] The optical

Table 2. ^{1}H NMR data for α and β isomers of methyl L-kedarosaminide.[a]

Position	Methyl α-L-kedarosaminide resonance	Methyl β-L-kedarosaminide resonance
1	4.81 (t)	4.34 (dd)
2 (eq)	1.90 (ddd)	2.04 (ddd)
2 (ax)	1.77 (ddd)	1.61 (ddd)
3	3.95 (dt)	3.72 (dt)
4	2.51 (m)	2.47 (m)
5	4.11 (ddd)	3.68 (ddd)
6	1.41 (d)	1.46 (d)
N–CH_3	2.61 (s)	2.65 (s)
O–CH_3	3.31 (s)	3.48 (s)

[a] Chemical shifts were measured in $CDCl_3$ solution and expressed in ppm downfield from internal TMS.

rotation of the predominantly methyl α-glycoside obtained from A82548A was very close to that published for the pure isomer obtained by total synthesis, indicating that the amino sugar in A82548A was also L-kedarosamine.[18] This result thereby established the absolute stereochemistry of A82548A as that depicted in Fig. 3.

NMR Assignments

After the full structural details and absolute stereochemistry of A82548A had been established by X-ray crystallography, the ^1H and ^{13}C NMR assignments were made for this compound. The previous assignments made for cytovaricin were useful, although a couple of differences with the published literature values were found, as noted below.[8] The NMR assignments have also been correlated with those for ossamycin, another related spiroketal macrolide.[14]

In both $CDCl_3$ and acetone-d_6 as solvent, two components of A82548A were observed that were presumed to be due to the mixture of anomers at C-34. In $CDCl_3$ solution, the initial ratio of the two components was approximately 3:1, but upon standing over a two-day period, the ratio changed to approximately 5:1. The assignments given in Table 1 were obtained from this 5:1 solution. The 1D ^{13}C NMR spectrum was first obtained and the various resonances were assigned to methyl, methylene, methine, and quaternary carbon atoms by use of DEPT experiments. The ^{13}C spectrum was then correlated to the ^1H spectrum using HMQC (one bond heteronuclear correlation). An HMBC (long range $^{13}C/^1H$ correlation) experiment was then conducted and the assignments for many of the positions were made through an analysis of this data (see Fig. 4 for some of the long range correlations observed). The assigned resonances were confirmed and the positions not yet assigned from the HMBC data were then determined by use of COSY, TOCSY, and HMQC-TOCSY experiments. Based upon long range heteronuclear correlations between the pairs: H-44/C-7, H-5/C-8, and H-7/C-8, our assignments for C-7 and C-31 were found to differ from the published values for cytovaricin.[9] This observation has also been found in the NMR data for ossamycin.

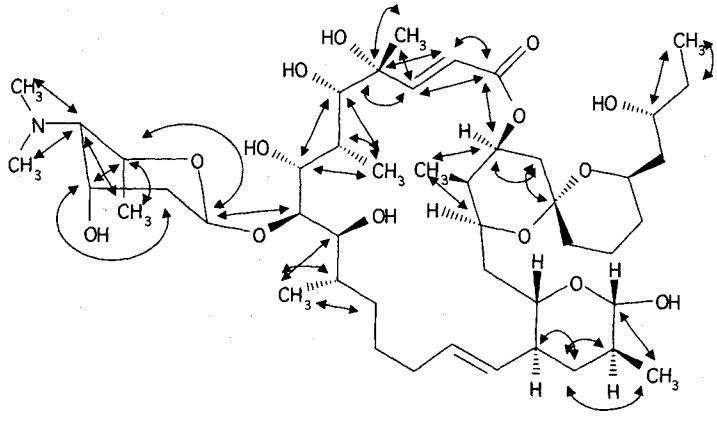
The coupling of H-33 with H-34 is 8.1 Hz, a value that indicates a *trans* diaxial relationship between these two protons. Consequently, we conclude that the more stable conformation of the anomeric hydroxyl group of A82548A in $CDCl_3$ solutions is *trans* to the methyl group at C-33 (Fig. 4).

Discussion

A82548A adds a new structural variation to the polyketide-derived family of spiroketal-containing macrolides that include cytovaricin, oligomycin, rutamycin, ossamycin, kaimonolide, and phthoramycin. This report also extends the known relative and absolute stereochemical patterns to an additional member of this series, indicating the high degree of consistency being found in the stereochemistry within this family of macrolides. The absolute stereochemistry of A82548A at each of its asymmetric centers is identical to that of cytovaricin, as depicted by EVANS and co-workers; thus, A82548A completely fits the stereochemical patterns described for this family of macrolides.[12,13] Although the hemiketal moiety of A82548A differs from that of cytovaricin, this same hemiketal has been recently described in SS49,[12] indicating that either of these two different hemiketal systems can be assembled *via* secondary metabolism within various microorganisms.

It is also of interest to note that even though different microorganisms have synthesized the same amino sugar, L-kedarosamine, it is linked to completely different types of aglycones that bear no resemblance to each other.

Fig. 4. Long range correlations for A82548A from HMBC experiments.



Furthermore, the conformation of kedarosamine adopted within each compound is different, as illustrated in Fig. 5. In A82548A, the anomeric bond and dimethylamino groups are equatorial and the methyl and hydroxyl groups are axial, whereas the exact opposite is observed in kedarcidin.[17] An L-sugar possessing an equatorial anomeric bond had been first described for L-megosamine in the macrolide antibiotic, megalomicin.[20] The preference for either an axial or an equatorial anomeric linkage is most likely explained by differences in the intra- and intermolecular environments surrounding the particular sugar, in which different hydrogen bonding arrangements, lipophilic/hydrophilic interactions, and steric constraints favor one conformation over the other within each molecule.

The 1,3-diaxial interaction between the C-3 hydroxyl group and C-5 methyl group of kedarosamine in A82548A causes these two groups to repel and spread apart from each other. Examination of the X-ray data indicates that these groups are not parallel, but rather form an angle of 22° between the C(3)-OH and C(5)-CH_3 bonds as a result of the distortion necessary to relieve the unfavorable 1,3-diaxial interaction.

NMR studies of kedarosamine in A82548A have confirmed that its solution conformation is the same as its solid state conformation. In this case, the NMR spectra were measured in acetone-d_6 solution because the desired resonances were better resolved in this solvent. Proton resonances of kedarosamine were located by homonuclear decoupling and the coupling constants were measured (Table 3), which were all completely consistent with the stereochemistry assigned to kedarosamine in A82548A. Furthermore, the observed NOEs between the anomeric proton and H-8 and 10-CH_3 of the aglycone indicated that the saccharide adopts the same orientation of the ring in the solution and solid states. These studies have firmly established the structure of A82548A as that depicted in Figs. 2 and 3.

Fig. 5. Conformational difference of kedarosamine in A82548A and kedarcidin.

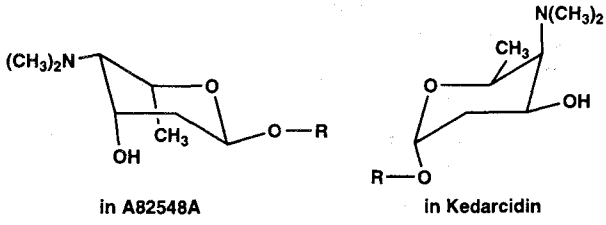


Table 3. ^{1}H NMR data for the kedarosamine moiety within A82548A.[a]

Position	Proton resonance	Coupling constants	ROE
35	5.06	7.3 (H-2_{ax}) 2.7 (H-2_{eq})	H-2_{eq}, H-6 Ring H-8, Ring 10-CH_3
36 (eq)	2.06	5.7 (H-3)	H-3
36 (ax)	1.72	3.7 (H-3)	H-3
37	4.23	3.4 (H-4)	H-4, $N(CH_3)_2$
38	2.23	4.6 (H-5)	H-5
39	4.42	7.0 (H-6)	H-6, $N(CH_3)_2$
40	1.44		
41, 42	2.37		

[a] Chemical shifts were measured in acetone-d_6 solution and expressed in ppm downfield from internal TMS. Coupling constants are measured in Hz.

Experimental

General Methods

NMR spectra were obtained on a GE QE-300 spectrometer in $CDCl_3$ solution at room temperature. Optical rotations were measured on a JASCO model DIP-70 polarimeter. Infrared spectra were determined on a Nicolet 510P spectrometer. FAB and high resolution mass spectrometry were conducted on a VG-Analytical ZAB-2-SE spectrometer. X-ray crystallographic data were collected with a Siemens R3M diffractometer using monochromated CuKα radiation ($\lambda = 1.5418$ Å).

Isolation of A82548A

Fermentation broth (200 liters) was filtered using a filter aid (2% Hyflo Supercel, Johns-Mansville Products) and the residual biomass was extracted with methanol (50 liters) and filtered as before. This filtrate was concentrated to aqueous under reduced pressure to approximately 6 liters and was then extracted three times with equal volumes of ethyl acetate. These combined extracts were evaporated under reduced pressure to give a solid residue (6.6 g), which was dissolved in chloroform (20 ml) and applied to a column of silica gel (500 g, E. Merck grade 62, 60~200 mesh), eluting with chloroform-methanol (99.5 : 0.5, 1.4 liters; then 9 : 1, 700 ml). The eluate from the 9 : 1 solvent mixture was concentrated under reduced pressure to a brown oil (3.3 g), which was further purified by chromatography on Sephadex LH-20 (2200 g, 20~100 μm, Pharmacia Fine Chemicals), using methanol as eluent. After discarding the first 500 ml, 100 ml fractions were collected and analyzed by HPLC (3.9 × 300 mm μBondapak C18 column (Millipore), mobile phase of acetonitrile-water (47 : 53) containing 0.2% triethylamine adjusted to pH 3.2, UV detection at 225 nm). Factor A was found in fractions 4~6, which were pooled and concentrated to a pale yellow oil (1.1 g). This oil was dissolved in methanol (20 ml) and the apparent pH was raised from 7.8 to 9.2 with 5% KOH (w/v); water was added dropwise with stirring until crystallization was complete (approximate final volume of 300 ml). The colorless crystals were collected by filtration and dried *in vacuo*, yielding pure A82548A (725 mg): mp 198~200°C; $[\alpha]_D^{22}$ −62.6° (*c* 1.99, MeOH); pK_a 8.0 (66% DMF), IR ($CHCl_3$) 3550, 3380, 3021, 2844, 1711 cm^{-1}; UV (EtOH) 214 nm (ε 11,200); NMR: see Table 1; FAB-MS 884 (MH^+); *Anal.* Calcd. for $C_{47}H_{81}NO_{14}$: C 63.85, H 9.23, N 1.58. Found: C 63.71, H 9.31, N 1.59.

Methyl L-Kedarosaminide from Hydrolysis of A82548A

A82548A (1.51 g, 1.7 mmol) was suspended in methanol (30 ml) and treated with a solution of HCl in methanol (50 ml), which had been generated by bubbling dry HCl into ice-cold methanol for 15 minutes. The mixture was capped and stirred at room temperature for 5 hours, during which it turned a dark color. The mixture was diluted with water and washed with dichloromethane to remove organic-soluble material. The aqueous acidic solution was made basic with 5 N NaOH, saturated with solid NaCl, and extracted with dichloromethane; the extract was dried over anhydrous K_2CO_3 and evaporated at room temperature *in vacuo*, yielding 211 mg of a light yellow oil. The product was purified by bulb-to-bulb distillation (100°C, 0.5 torr) to yield 176 mg (55%) of methyl L-kedarosaminide in a 7:1 mixture of $\alpha:\beta$ anomers (by NMR) as a colorless oil; $[\alpha]_D^{22}$ −140.5° (*c* 0.73, $CHCl_3$) (lit.[18] $[\alpha]_D^{22}$ −149.5° (*c* 1.04, $CHCl_3$) for the pure α-anomer); IR (neat) 2932, 2832, 1467, 1448, 1407, 1384, 1358 cm^{-1}; NMR (see Table 2); FAB-MS *m/z* 190 (MH^+, 100); Exact Mass HR-FAB-MS *m/z* 190.1453, calcd for $C_9H_{20}NO_3$: $(MH)^+$ 190.1443.

X-ray Crystallographic Studies

A suitable crystal for data collection was obtained by diffusing water into an ethanolic solution of A82548A. A colorless crystal with dimensions of 0.20 × 0.30 × 0.30 mm^3 was mounted in a glass capillary, cooled to −100°C, and used for all subsequent experiments. Preliminary diffraction photographs showed monoclinic symmetry. Systematic extinctions and a least-squares fit of 25 reflections ($35° \leq 2\theta \leq 45°$) defined space group $P2_1$ with unit cell dimensions a = 9.949 (3), b = 20.823 (11), c = 24.823 (14) Å, and $\beta = 94.83\,(4)°$. The cell volume of 5124 (4) $Å^3$ corresponds to two $C_{47}H_{81}O_{14}N \cdot C_2H_5OH$ units (Z = 4) in the asymmetric unit and a calculated density $\rho_{calc} = 1.199\ g/cm^3$. Intensities for 6693 unique reflections ($2\theta \leq 110°$) were measured using $\theta:2\theta$ scans with a variable scan speed (2.0 ~ 29.30°/minute). Of these, 5893 (88%) were judged observed [$|F_o| \geq 2\sigma(F_o)$]. The data showed no intensity decay and were corrected for Lorentz and polarization effects.

A successful structure solution was obtained by the implementation of direct methods (SHELXTL).[21] Full-matrix least-squares refinements (SHELXL93)[22] for anisotropic nonhydrogen atoms and isotropic rigid hydrogen atoms converged to a final crystallographic residual $R_1 = 6.3\%$, $wR_2 = 16.4\%$ (on F^2). A final difference Fourier map revealed a maximum residual density of 0.39 $e^-/Å^3$.

Acknowledgments

We greatly appreciate the assistance of the Lilly physical chemistry department for physicochemical and spectral data, Mr. LA VERNE BOECK for fermentation support, and Ms. L. W. CRANDALL for literature searches. We thank Dr. JOHN LEET of Bristol-Myers Squibb Company and Prof. FERENC SZTARICSKAI of the Hungarian Academy of Sciences for helpful discussions about kedarcidin and kedarosamine. We also thank Dr. GABOR LUKACS of the C.N.R.S., Gif-sur-Yvette, for pointing out the historical precedent of β-L-megosamine in megalomicin.

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