J. Med. Chem. **1997,** *40,* 1041−1045

Notes

Repromicin Derivatives with Potent Antibacterial Activity against *Pasteurella multocida*

James W. McFarland,* Scott J. Hecker,† Burton H. Jaynes, Martin R. Jefson, Kristin M. Lundy, Chi B. Vu,‡ Edward A. Glazer, Susan A. Froshauer, Shigeru F. Hayashi, Barbara J. Kamicker, Catherine P. Reese, and Julie A. Olson

Central Research Division, Pfizer Inc., Groton, Connecticut 06340

Received June 13, 1996⊗

Reductive amination of repromicin with polyfunctional amines has led to new macrolide antibacterial agents, some of which are highly potent against the Gram-negative pathogen *Pasteurella multocida* both in vitro and in a mouse infection model. A key element in this discovery was the recognition that among certain known macrolides increasing lipophilicity results in diminished in vivo activity. One repromicin derivative, 20-{*N*-[3-(dimethylamino)-propyl]-*N*-L-alanylamino}-20-deoxorepromicin (**35**), was selected for advanced evaluation. At 5 mg/kg, a single subcutaneous dose was found to control induced pasteurellosis in swine and induced respiratory disease in cattle.

Bovine respiratory disease (BRD) causes heavy losses in the cattle industry. These include unthrifty weight gains, the deaths of calves, and the cost of treatment. Treatment costs consist of drugs and labor in handling the animals. Thus, single-dose treatment, requiring less work, has an economic advantage. Tilmicosin,[1] a desmycosin derivative introduced into the U.S. market in 1990, is effective against BRD at 10 mg/kg as a single subcutaneous injection and has gained acceptance rapidly. Tilmicosin owes its single-dose effectiveness to its long half-life and its high concentration in lung tissue.[2]

Pasteurella haemolytica and *Pasteurella multocida* are the main bacterial components of the BRD complex. Control of these Gram-negative pathogens normally overcomes the illness. While macrolides are generally considered to be agents for Gram-positive infections, there is precedence for activity against nonenteric Gram-negative bacteria. For example, azithromycin is effective against human obstructive pulmonary disease due to *Haemophilus influenza*.[3]

We now report the discovery of new macrolide antibacterial agents. Reductive amination derivatives of repromicin[4,5] are active against *Pasteurella* species, among others, in vitro. Several of this group are highly potent in mouse protection studies.

Workers at Eli Lilly and Company investigated a wide variety of tylonolides in addition to desmycosin but did not report anything significantly better than tilmicosin.[6−9] We initiated our studies with the relatively unexplored macrolide rosaramicin because it is more potent against *P. multocida* in vitro than is tilmicosin and has comparable mouse protective activity. When reductively aminated with 3,5-dimethylpiperidine, rosaramicin afforded the analog **1**. While **1** retained the in vitro potency of its parent, it had no in vivo activity at up to 150 mg/kg. We sought an explanation for this surprising finding.

By studying in vitro data published on reductive amination products of desmycosin,[6,7] we observed a tendency for the more lipophilic compounds to be the more potent. The relative lipophilicities in this series were estimated simply by counting the number of carbons in the amine moiety. Optimal potency appears to be at six or seven carbon atoms. The situation with in vivo activity was much less clear.

Thus, we considered lipophilicity to be potentially a key property in determining in vivo activity, but in comparing macrolides across different series, counting carbon atoms was no longer reasonable. To overcome this difficulty, we used reversed phase HPLC retention times to estimate relative lipophilicities. In this work these values were transformed into the logarithms of their capacity factors (log k') by the formula:

$$\log k' = \log(t_R/t_0 - 1) \quad (1)$$

where t_R is the retention time of the analyte and t_0 is the column dead time. It has been shown that log k' is proportional to log P.[10]

Initially, we chose nine macrolides that seemed relevant to the problem at hand. These compounds are listed in Table 1 in order of their increasing log k's. As shown in the table there is no apparent connection among these compounds between MIC and lipophilicity. However, it is apparent that those compounds with log k's > 0.7 are not active in vivo, but those with lesser log k's are. Hence, the lack of in vivo activity appears to be associated with high lipophilicity, a somewhat counterintuitive concept in view of the relationship just noted with in vitro potency among desmycosin derivatives. This observation led to the discovery of in vivo active derivatives among rosaramicin analogs (see below).

* To whom correspondence should be addressed. Tel: (860) 441-3692. Fax: (860) 441-6854 [call (860) 441-3692, indicate a message has been sent]. E-mail: mcfarland@pfizer.com.

† Present address: Microcide Pharmaceuticals, Inc., 850 Maude Ave, Mountain View, CA 94043.

‡ Present address: Ariad Pharmaceuticals, Inc., 26 Landsdowne St, Cambridge, MA 02139.

⊗ Abstract published in *Advance ACS Abstracts,* February 1, 1997.

S0022-2623(96)00435-9 CCC: $14.00 © 1997 American Chemical Society

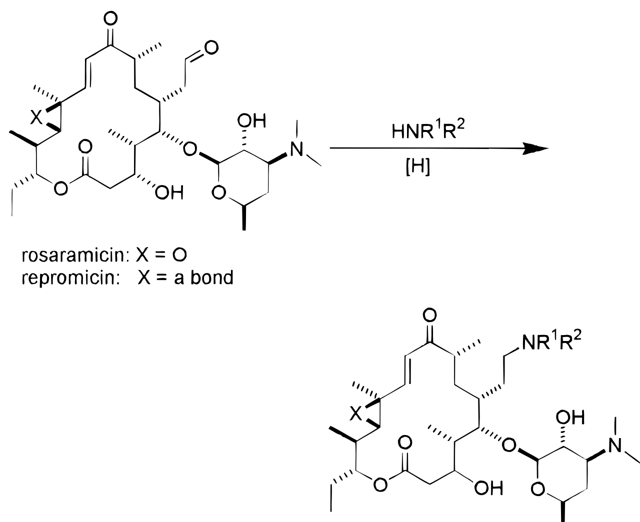
Table 1. Selected Macrolides: In Vitro and in Vivo Activities against *P. multocida* 56A006 and HPLC Capacity Factors (log *k*′)

macrolide[a]	MIC (μg/mL)	PD_{50} (95% CL) (mg/kg)	log *k*′ [b]
desmycosin	3.13	66 (44−99)	0.17
tilmicosin	1.56	99[c]	0.26
azithromycin	0.05	16 (13−19)	0.38
rosaramicin	0.20	68 (47−107)	0.63
tylosin	25.00	86 (64−119)	0.68
1	0.20	>150	0.79
repromicin	0.39	>200	0.86
carbomycin A	1.56	>150	1.48
carbomycin B	6.25	>150	1.76

[a] Ranked in order of increasing log *k*′. [b] As determined by HPLC method I (see the Experimental Section). [c] The PD_{50} value for tilmicosin is an estimate owing to the toxicity of this compound to mice in this dose range.

Chemistry

The new compounds described here were prepared by reductively aminating the aldehyde function at the C-20 positions of rosaramicin and repromicin as illustrated in the scheme below. Methods using formic acid and $NaBH_3CN$ and its variants as reductants were adapted from the work of Debono et al.[7] Generally, the formic acid procedure worked well for secondary amines but was less effective with primary amines. The reverse was true for reductive amination using the borohydrides. In the later stages of this work, amino acids were conjugated to several of the reductive amination products described here. These were prepared by standard procedures for this type of compound. The methods are exemplified in the Experimental Section.



Results and Discussion

We reduced rosaramicin with amines containing fewer carbon atoms than 3,5-dimethylpiperidine. The results are summarized in Table 2. As shown, the somewhat less lipophilic piperidine and diethylamine derivatives afforded mouse protection with the same order of potency as that of tilmicosin. However, as the number of carbon atoms in the amine decreased further, in vivo potency was reduced. Hence, in this series of simple alkylamines there is an optimum range of lipophilicity associated with in vivo activity.

We did the same with repromicin. The results are shown in Table 3. In general, we obtained lower MIC's and greater in vivo potency with repromicin derivatives than with those of rosaramicin. For this reason, we continued our studies almost exclusively with repromicin. In comparing the rosaramicin and chemically alike repromicin derivatives, we noticed that the number of carbon atoms necessary for optimal in vivo potency is lower for the latter series. Undoubtedly, this is a reflection of the fact that repromicin is more lipophilic than rosaramicin. This observation is confirmed by comparing the log *k*′s listed in Tables 2 and 3.

To pursue the idea that lower lipophilicity would lead to still better in vivo potency, we prepared derivatives from polyfunctional amines. Two approaches were followed initially. The first was to use β-hydroxyamines to obtain even more hydrophilic products; the second was to use diamines to furnish an additional electronic charge on the molecule, i.e., another means to increase polarity. Later, we found that amino acid conjugates of certain diamine products often gave enhanced in vivo potency. As indicated by many of the compounds **18**−**38**, we were amply rewarded for our efforts. For example, in Table 3 there are 12 analogs more potent than azithromycin, namely, compounds **19**, **23**, **25**, **27**, **30**−**32**, and **34**-**38**.

Finally, we applied these discoveries to the desmycosin series. As shown in Table 4, additional basic sites did not result in any significant improvements in MIC's or PD_{50}'s.

To some extent these observations are related to the principle of minimal hydrophobicity introduced by Hansch, Björkroth, and Leo.[11] In essence, the principle suggests that high hydrophobicity is commonly a positive factor for obtaining potent activity in cell-free, single-cell, or tissue culture systems, yet this very same property may result in undesirable pharmacokinetics in whole animals. Thus, we need a minimal degree of hydrophobicity to maintain intrinsic potency but not so much as to lead to unfavorable in vivo activity. In the case of the macrolides described here, we see something quite similar.

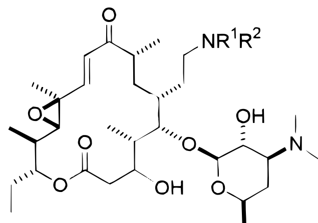
From these investigations we discovered several repromicin derivatives of potential commercial caliber. Among these compounds **35** was selected for further evaluation. At a single dose of 5 mg/kg, **35** effectively controlled induced pasteurellosis in swine and induced BRD when administered by the subcutaneous route.[12]

Experimental Section

Common Synthetic and Characterization Procedures. Compounds were prepared by the methods illustrated below. In general each new derivative was isolated by quenching the reaction with saturated aqueous $NaHCO_3$ and extracting the product with EtOAc, CH_2Cl_2, or $CHCl_3$. The extract was dried over anhydrous Na_2SO_4 or $MgSO_4$, filtered, and evaporated under reduced pressure to furnish crude material that was then purified by chromatography on silica gel. The eluent was typically CH_2Cl_2−MeOH−concentrated aqueous NH_4OH (v:v:v, 90:10:1). Purity was confirmed by HPLC. Because few attempts were made to optimize them, the yields ranged widely, about 10−97%. Once a pure compound was in hand, it was characterized by its HPLC retention time (see below), field desorption mass spectrometry (nominal molecular ion plus a proton, i.e., M + 1), and NMR spectroscopy, both ^1H and ^{13}C. For each reported compound, the spectral properties agreed with the assigned structure.

High-Performance Liquid Chromatography (HPLC). Method I. Determination of Retention Times for log *k*′. Retention times were determined on a YMC 5 μm C-8 column (4.6 mm i.d. × 250 mm length) from Eicon Scientific (P.O. Box

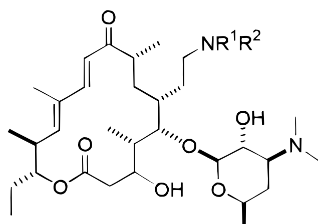
Table 2. Rosaramicin Derivatives: In Vitro and in Vivo Activities against *P. multocida* 56A006 and HPLC Capacity Factors (log *K*′)



compd	NR^1R^2	synth method	purity[a] (%)	log *K*′ [b]	*P. multocida* 59A006 MIC (μg/mL)	*P. multocida* 59A006 PD_{50} (95% CL) (mg/kg)
1	3,5-dimethylpiperidin-1-yl	A	96[c]	0.79	0.20	>150
2	hexahydroazepin-1-yl	A	95	0.54	0.20	150[d]
3	piperidino	A	92	0.40	0.20	114 (77−210)
4	diethylamino	A	96	0.35	0.78	123 (91−166)
5	pyrrolidino	A	93	0.32	0.78	>150
6	dimethylamino	A	94	0.27	0.39	>150

[a] As determined by HPLC method II (see the Experimental Section). [b] As determined by HPLC method I (see the Experimental Section). [c] As a 92:8 mixture of *cis:trans* isomers. [d] The highest dose tested; 4−6 out of 10 mice in the treatment group survived; no survivors at lower doses; hence, this is an approximate PD_{50}.

Table 3. Repromicin Derivatives: In Vitro and in Vivo Activities against *P. multocida* 56A006 and HPLC Capacity Factors (log *K*′)

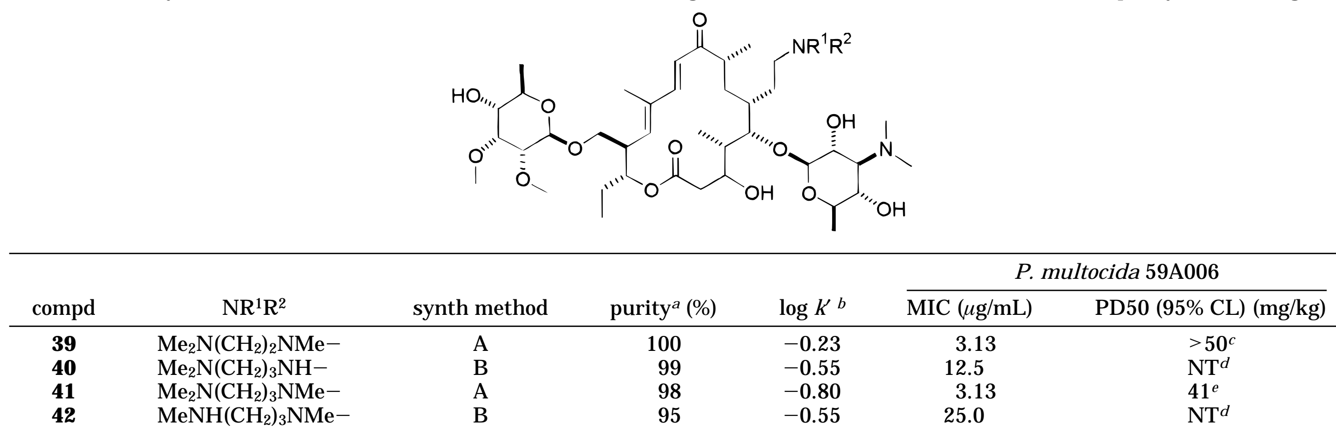


compd	NR^1R^2	synth method	purity[a] (%)	log *K*′ [b]	*P. multocida* 59A006 MIC (μg/mL)	*P. multocida* 59A006 PD_{50} (95% CL) (mg/kg)
7	hexahydroazepin-1-yl	A	100	0.64	0.10	>200
8	piperidino	A	90	0.50	0.20	150[c]
9	diethylamino	A	100	0.47	0.39	141 (98−203)
10	pyrrolidino	A	94	0.42	0.39	128 (77−212)
11	ethylmethylamino	A	99	0.38	0.20	150[c]
12	azetidin-1-yl	A	100	0.27	0.20	46 (31−72)
13	dimethylamino	A	98	0.30	0.39	150[c]
14	morpholino	A	100	0.64	0.78	>150
15	4-methylpiperazin-1-yl	A	100	0.44	0.39	>150
16	ethylamino	B	93	0.24	0.20	>150
17	*tert*-butylamino	B	94	0.66	0.39	150[c]
18	3-hydroxyazetidin-1-yl	B	98	0.15	0.20	19 (4.9−70)
19	$HOCH_2CH_2NH-$	B	88	0.10	0.20	10 (6.6−15)
20	$HOCH_2CH_2NMe-$	A	99	0.16	0.20	45[d]
21	$(HOCH_2CH_2)_2NH-$	A	97	ND[e]	0.20	41 (29−57)
22	$HOCH_2CMe_2CH_2NH-$	B	99	ND[e]	0.05	26 (21−32)
23	$HOCH_2CH(OH)CH_2NH-$[f]	B	96	0.01	0.39	10 (7.3−13)
24	$(HOCH_2)_2CHNH-$	B	86	ND[e]	0.39	31 (24−41)
25	2,6-dihydroxycyclohexylamino[g]	B	97	0.23	0.20	11 (3.5−36)
26	$Me_2NCH_2CH_2NH-$	B	91	0.37	0.20	100[c]
27	$Me_2N(CH_2)_3NH-$	C	100	0.19	0.39	5 (3.6−6.2)
28	$Me_2N(CH_2)_3NMe$-	A	100	ND[e]	0.10	26 (21−32)
29	$MeNHCH_2CH_2NMe-$	C	100	0.27	0.20	>20[h]
30	$MeNH(CH_2)_3NMe-$	B	88	-0.05	0.78	4 (2.5−5.3)
31	Gly-$NMeCH_2CH_2NMe-$	D	100	-0.10	0.20	4 (3−7)
32	L-Ala-$NMeCH_2CH_2NMe-$	D	100	0.02	0.05	12 (5.8−24)
33	$Me_2N(CH_2)_3N(Ac)-$	E	100	0.22	0.39	>25[h]
34	$Me_2N(CH_2)_3N$(Gly)−	D	100	-0.25	0.78	4 (3.0−6.5)
35	$Me_2N(CH_2)_3N$(L-Ala)−	D	97	0.12	0.39	3 (1.9−3.9)
36	$Me_2N(CH_2)_3N$(sarcosyl)−	D	100	ND[e]	1.56	6[d]
37	L-Ala-$NMe(CH_2)_3NMe-$	D	100	-0.16	0.39	5 (3.6−6.2)
38	sarcosyl-$NMe(CH_2)_3NMe-$	D	100	-0.06	0.39	6 (4.1−9.9)

[a] As determined by HPLC method II (see the Experimental Section). [b] As determined by HPLC method I (see the Experimental Section). [c] The highest dose tested; 4−6 out of 10 mice in the treatment group survived; no survivors at lower doses; hence, this is only an approximate PD_{50}. [d] No confidence interval is reported when only two doses were tested. [e] Not determined. [f] Mixture of epimers. [g] Hydroxy groups *cis* to each other and *trans* to the amino group. [h] Highest dose tested.

70, Medway, MA). The eluent was a 35:65 (v:v) mixture of MeCN−aqueous NH_4OAc (50 mmol). The column was maintained at room temperature and the flow rate at 1.0 mL/min. Each sample was dissolved in the premixed eluent solvent (1

Table 4. Desmycosin Derivatives: In Vitro and in Vivo Activities against *P. multocida* 56A006 and HPLC Capacity Factors (log *k*′)



compd	NR^1R^2	synth method	purity[a] (%)	log *k*′ [b]	*P. multocida* 59A006 MIC (μg/mL)	PD50 (95% CL) (mg/kg)
39	$Me_2N(CH_2)_2NMe-$	A	100	−0.23	3.13	>50[c]
40	$Me_2N(CH_2)_3NH-$	B	99	−0.55	12.5	NT[d]
41	$Me_2N(CH_2)_3NMe-$	A	98	−0.80	3.13	41[e]
42	$MeNH(CH_2)_3NMe-$	B	95	−0.55	25.0	NT[d]

[a] As determined by HPLC method II (see the Experimental Section). [b] As determined by HPLC method I (see the Experimental Section). [c] Highest dose tested. [d] Not tested. [e] No confidence interval is reported when only two dose levels were tested.

mg/mL) and injected via an LDC 713 autosampler (Thermo Separation Products, 3661 Interstate Park Rd N, Riviera Beach, FL) into an air-actuated Rheodyne 7126 (Cotati, CA) injection valve equipped with an injection loop (20 μL). The pumping system was an LDC CM 4000. Peaks corresponding to the sample input were detected by UV spectroscopy at 254 nm (rosaramicin derivatives) or 280 nm (repromicin derivatives) with an LDC SM 3100 UV detector.

High-Performance Liquid Chromatography (HPLC). Method II. **Determination of Compound Purity.** Retention times and areas-under-the-curve (AUCs) were determined on a YMC 5 μm C-8 column (4.6 mm i.d. × 250 mm length) from Eicon Scientific (P.O. Box 70, Medway, MA), different from the one described above, but manufactured to the same specifications. The eluent was a 30:70 (v:v) mixture of MeCN−aqueous NH_4OAc (50 mmol). The column was maintained at room temperature and the flow rate at 1.0 mL/min. Each sample was dissolved in the premixed eluent solvent (1 mg/mL) and injected into a Hewlett Packard 1050 system equipped with a 40 μL injection loop. An evaporative light scattering detector (ELSD; Alltech/Varex Model MK III) was used in this assay because it responds more accurately to the mass distribution of the peaks than does a UV-based system.

Method A. **Reductive Amination with Formic Acid. 20-Azetidin-1-yl-20-deoxorepromicin (12).** This method was adapted from the work of Debono et al.[7] A solution of repromicin (6.61 g, 11.69 mmol), azetidine (1.00 g, 17.51 mmol), and 225 mL of EtOAc was heated to 70 °C with stirring. Formic acid (0.591 g, 12.8 mmol) was added dropwise to the solution, and the temperature was lowered to 65 °C. Stirring and heating was continued for 5 h. After cooling to room temperature, the reaction solution was washed twice with 250 mL portions of saturated aqueous $NaHCO_3$ and then once with 200 mL of saturated aqueous NaCl. The combined extracts were dried over anhydrous $MgSO_4$, filtered, and evaporated under reduced pressure to furnish 6.56 g of crude product as a yellow foam. This material was taken up in 100 mL of hot Et_2O; insolubles were filtered and saved. The filtrate was treated with 300 mL of hot hexane, and again the resulting insoluble matter was filtered and saved. The filtrate was concentrated to about 75 mL by boiling off excess solvent. The resulting solution was allowed to cool to room temperature and then cooled to 5 °C for several hours. A colorless precipitate of **12** formed, yield 2.58 g (36%). The filtrate was combined with the insolubles that were saved, and the mixture was then chromatographed on 450 cc of silica gel. Elution with 9:1 CH_2Cl_2−MeOH containing 1% NH_4OH afforded 1.88 g (27%) of additional product for a total yield of 63% of **12** free base.

Method B. Reductive Amination with $NaBH_3CN$. 20-(Ethylamino)-20-deoxorepromicin (16). Under a N_2 atmosphere and with magnetic stirring, a solution of 223 mg (3.55 mmol) of $NaBH_3CN$, 50 μL (0.88 mmol) of ethylamine, and 10 mL of MeOH was treated dropwise with a solution of 500 mg (0.88 mmol) of repromicin in another 10 mL of MeOH. The reaction mixture was worked up in the manner described in the beginning paragraph of this section. There was obtained 96 mg (18%) of **16**.

Method C. Reductive Amination with $NaBH(OAc)_3$. 20-[2-(Dimethylamino)ethyl]-20-deoxorepromicin (26). To a magnetically stirred solution of 1.5 g (2.65 mmol) of repromicin in 8 mL of MeOH was added 0.61 mL (5.3 mmol) of *N*,*N*-dimethylethylenediamine. After being stirred at room temperature for 30 min, the solution was treated with 0.15 mL (2.65 mmol) of HOAc and cooled to 0 °C. In 2 mL of MeOH, 167 mg (2.66 mmol) of $NaBH_3CN$ was then added over a period of 10 min. Stirring and cooling was continued for 10 min. The reaction mixture was worked up in the manner described in the beginning paragraph of this section. The crude product was chromatographed on silica gel (eluent CH_2Cl_2−MeOH, 90:10, to CH_2Cl_2−MeOH−TEA, 88:12:0.5) to furnish 650 mg (38%) of **26**. Anal. ($C_{35}H_{63}N_3O_7$) C, H, N.

Method D. Acylation with *t*-BOC-Protected Amino Acids followed by Deprotection. 20-{*N*-[3-(Dimethylamino)propyl]-*N*-L-alanylamino}-20-deoxorepromicin Sesquihydrate (35). At 0 °C, a solution of 4.1 g (21.8 mmol) of *N*-*t*-BOC-L-alanine, 4.5 g (21.8 mmol) of dicyclohexylcarbodiimide, and 2.95 g (21.8 mmol) of hydroxybenzotriazole in 109 mL of CH_2Cl_2 was treated with 14.2 g (21.8 mmol) of **27**. The mixture was allowed to warm to room temperature and was stirred for 3 days. It was then filtered, and the filtrate was evaporated under reduced pressure to furnish a crude product. The crude material was chromatographed on silica gel (gradient elution, 2% MeOH−0.25% NH_4OH in $CHCl_3$ to 4% MeOH−0.5% NH_4OH in $CHCl_3$) to afford 10.02 g (53%) of BOC-protected **35**. At 0 °C, a solution of this material in 100 mL of CH_2Cl_2 and CF_3CO_2H (1:1) was stirred for 20 min. The volatile components were evaporated under reduced pressure, and the residue was triturated with Et_2O and filtered. The product was taken up in H_2O; the solution was adjusted to pH 10 with 1 N NaOH, extracted with $CHCl_3$, dried over anhydrous Na_2SO_4, and concentrated to afford 8.59 g (97%) of **35**. Anal. ($C_{39}H_{70}N_4O_8 \cdot 1.5H_2O$) C, H, N.

Other agents, such as propylphosphonic anhydride or diethyl cyanophosphonate, in place of dicyclohexylcarbodiimide−hydroxybenzotriazole were also effective in this procedure.

Method E. Acetylation. 20-{*N*-[3-(Dimethylamino)propyl]-*N*-acetylamino}-20-deoxorepromicin (33). At room temperature, a solution of 600 mg (0.9 mmol) of **27**, 85 μL (0.9 mmol) of Ac_2O, and 4.5 mL of acetone was stirred for 2 h. The reaction solution was evaporated under reduced pressure to furnish 660 mg of crude product which was then chromatographed on silica gel. There was obtained 507 mg (81%) of **33**.

In Vitro Activity. MIC's of macrolides were determined by a liquid dilution method in microtiter format. The inoculum was prepared by transferring a single colony of *P. multocida* (strain 59A006) from an overnight plate of brain heart infusion (BHI) agar into 5 mL of BHI broth and incubating at 37° C, allowing the bacteria to grow to optical density of 0.2 at 600

nm. Each test compound (1 mg) was dissolved in 125 μL of DMSO; serial 2-fold dilutions were made in BHI broth; final concentrations ranged from 200 to 0.098 μg/mL. The *P. multocida* culture was diluted with BHI broth and inoculated into microtiter wells to give a final cell density of approximately 10^4 cells/200 μL. Inoculated plates were incubated at 37 °C for 18 h. The MIC is defined as the lowest concentration of compound inhibiting 100% of visible bacterial growth as compared to uninoculated controls. Azithromycin was used as a positive control for each assay.

Mouse Protection Studies. Mice were allotted to cages (10/treatment group) upon their arrival and allowed to acclimate for a minimum of 48 h before use. Each mouse was inoculated intraperitoneally with 0.5 mL of a 3×10^3 cfu/mL suspension of *P. multocida* (strain 59A006) in BHI broth. In a well-functioning assay, all of the mice in the nonmedicated, infected group (the controls) died by the end of the test. Thirty minutes following challenge, each mouse was treated with the test compound by subcutaneous administration. The compounds were dissolved or suspended in a solution comprised of 1% aqueous carboxymethylcellulose, 0.05% methylcellulose, 0.9% NaCl, and 1% polysorbate 80 and then given at 0.2 mL/mouse. Additional treatments were given at 4 and 24 h after challenge. Azithromycin was used as a positive control. Animals were observed daily for 4 days (96 h). The number of survivors in each group was recorded. The PD_{50} is the calculated dose at which the test compound protects 50% of challenged mice from death owing to the *P. multocida* infection.

Acknowledgment. Kyle T. Blair, Shang-Poa Chang, Julia G. Cronin, John P. Dirlam, Nancy C. Elliott, Edward J. Fontaine, Alexander Hurd, Christopher S. Jones, David A. Koss, Mary A. LeMay, Susan C. Lilley, Maria L. Muzzi, Thomas N. O'Connell, Stephen F. Petras, Scott B. Seibel, and Annette M. Silvia made valuable technical contributions in advancing this work. We thank them for their fine efforts. Also, we thank Dr. Peter C. Canning of Pfizer Central Research, Terre Haute, IN, for allowing us to use some of his results from testing compound **35** in swine and cattle.

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JM960435Q