Identification and Synthesis of Products Isolated during Metabolism Studies of Tilmicosin

Herbert A. Kirst,*,† Alvin L. Donoho,‡ Lawrence C. Creemer,† Julie A. Wind,† Donnis M. Berry,† John L. Occolowitz,† and Jonathan W. Paschal†

Lilly Research Laboratories, Eli Lilly and Company, Lilly Corporate Center, Indianapolis, Indiana 46285, and Greenfield Laboratories, Eli Lilly and Company, Greenfield, Indiana 46140

Two substances, designated T-1 and T-2, were isolated during metabolism studies with tilmicosin, a new veterinary macrolide antibiotic. By combination of mass spectrometry and NMR spectroscopy, T-1 was identified as *N*-demethyltilmicosin and T-2 was assigned a dimeric structure resulting from reductive coupling of desmycosin with T-1. T-1 was synthesized by selective N-demethylation of tilmicosin, using $K_3Fe(CN)_6$ in aqueous 2-propanol. T-2 was synthesized by reductive amination of desmycosin with T-1 using $NaBH_3CN$. The synthetic compounds were identical with the respective isolated materials, thereby confirming the structural assignments for both compounds.

INTRODUCTION

Tilmicosin is a new veterinary macrolide antibiotic which has been recently approved for the treatment of bovine respiratory disease (BRD). It inhibits many bacteria that are pathogenic to animals, including species of *Pasteurella*, a causative agent of BRD (Ose, 1987). As a result of its long *in vivo* half-life, it is conveniently effective for both prophylaxis and treatment of respiratory diseases in cattle (Morck *et al.*, 1993; Laven and Andrews, 1991; Picavet *et al.*, 1991).

Tilmicosin is a semisynthetic derivative of tylosin, from which it is synthesized by an acid-catalyzed hydrolysis of the 2-deoxy sugar (mycarose) followed by reductive amination of the aldehyde group with 3,5-dimethylpiperidine (Kirst *et al.*, 1989; Debono *et al.*, 1989). During studies of the metabolism of tilmicosin, two compounds were isolated which were designated T-1 and T-2 (Donoho *et al.*, 1992). T-1 was identified as *N*-demethyltilmicosin and T-2 as a dimeric derivative of tilmicosin. In this paper, the structure and synthesis of the dimeric material T-2 are described; a convenient one-step N-demethylation procedure is applied to efficiently produce 3′-*N*-demethyltilmicosin (T-1).

MATERIALS AND METHODS

Synthesis of *N*-Demethyltilmicosin (T-1). A mixture of potassium ferricyanide (3.91 g, 11.9 mmol) and potassium hydroxide (847.7 mg, 15.1 mmol) was dissolved in water (50 mL) and cooled to 5 °C in an ice bath. Tilmicosin (5.0 g, 5.8 mmol) in 2-propanol (50 mL) was then added to this solution. The opaque biphasic mixture was stirred at 5 °C for 0.5 h and then allowed to warm to room temperature. After 6 h, the reaction was diluted with water and extracted with EtOAc. The extracts were washed with brine, dried (anhydrous K_2CO_3), and evaporated at room temperature under reduced pressure. The pale yellow solid (4.78 g) was chromatographed on silica gel, eluting with 0.5% NH_4OH in MeOH. Although separation was incomplete, a sample of pure *N*-demethyltilmicosin was isolated as a white glassy solid (2.08 g, 42%): IR ($CHCl_3$, cm^{-1}) 3436, 2975, 2933, 1592, 1457, 1378, 1314, 1236; FDMS *m/z* (relative intensity) 877 (10, M + Na^+), 855 (100, M + H^+), 838 (18), 679 (17). Anal. Calcd ($C_{45}H_{78}N_2O_{13}$): C, 63.21; H, 9.19; N, 3.28. Found: C, 63.35; H, 9.12; N, 3.30.

Synthesis of Dimeric Moiety T-2. *N*-Demethyltilmicosin (202.6 mg, 0.24 mmol) and desmycosin (177.9 mg, 0.24 mmol) were dissolved in methanol (3 mL) and stirred at room temperature for 20 min. $NaBH_3CN$ (23.6 mg, 0.38 mmol) was added

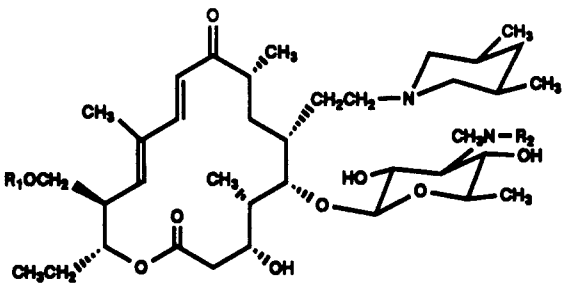
Table 1. NMR Data for T-1 (*N*-Demethyltilmicosin) and Tilmicosin in Acetone-d_6 at 500 MHz

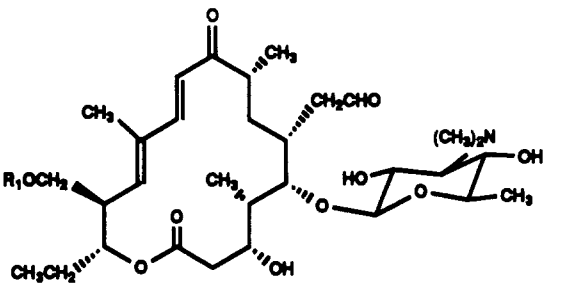
	^{13}C		^1H	
position	T-1	tilmicosin	T-1	tilmicosin
1	172.90	173.01		
2	40.13	40.25	2.30/1.98	2.30/1.98
3	66.98	67.35	3.76	3.76
4	42.82	42.77	1.58	1.57
5	79.72	80.03	3.62	3.59
6	32.42	32.86	1.62	1.62
7	34.52	34.53	1.75/1.46	1.70/1.48
8	45.96	45.89	2.59	2.59
9	203.67	203.62		
10	119.68	119.81	6.57	6.50
11	147.95	147.95	7.22	7.20
12	135.24	135.31		
13	143.27	143.07	5.91	5.87
14	45.77	45.77	2.97	2.95
15	74.64	74.79	4.98	4.97
16	25.60	25.65	1.93/1.64	1.91/1.61
17	9.99	9.95	0.94	0.92
18	9.25	9.30	0.99	0.97
19	25.07	25.21	1.71	1.69
20	55.20	55.38	2.75/1.98	2.72/1.98
21	18.15	18.15[b]	1.17	1.17
22	13.15	13.16	1.87	1.84
23	69.38	69.41	3.94/3.65	3.91/3.62
1′	105.31	105.58	4.25	4.25
2′	72.51	71.35	3.26	3.36
3′	66.98	71.73	2.35	2.34
4′	73.57	71.91	3.06	3.05
5′	73.90	73.94	3.33	3.28
6′	18.23[a]	18.21	1.23	1.19
NMe (s)	35.16	42.03	2.43	2.48
1″	102.06	102.05	4.56	4.55
2″	82.68	82.76	3.02	3.00
3″	81.42	81.41	3.74	3.72
4″	73.90	74.00	3.11	3.09
5″	70.77	70.32	3.56	3.55
6″	17.90[a]	17.92[b]	1.19	1.17
2″-OMe	59.45	59.41	3.47	3.45
3″-OMe	61.76	61.74	3.53	3.51
2‴, 6‴	64.39	64.32	2.66/1.38	2.66/1.30
	59.56	59.77	2.84/1.03	2.83/1.05
3‴, 5‴	30.78	30.86	1.81	1.78
	30.54	30.65	1.86	1.84
4‴	43.45	43.47	1.67/0.46	1.66/0.45
3‴, 5‴-Me	19.98	19.98	0.79	0.78
	19.88	19.89	0.97	0.95

[a,b] Assignments with the same superscript may be reversed.

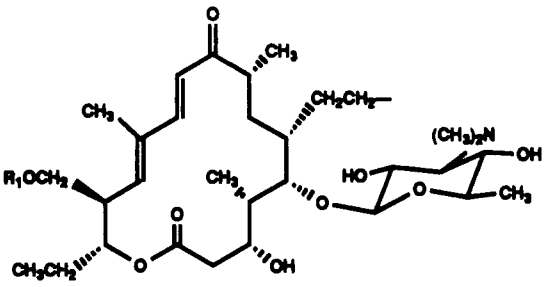
† Lilly Research Laboratories.
‡ Greenfield Laboratories.

0021-8561/94/1442-1219$04.50/0



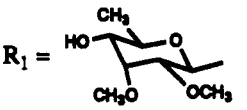


4 (Desmycosin)



1 (T-2): R_2 =

2 (T-1): R_2 = H



R_1 =

3 (Tilmicosin): $R_2 = CH_3$

Figure 1. Structures of T-1, T-2, tilmicosin, and desmycosin.

to this solution, and after stirring at room temperature for 3 h, solvent was evaporated under reduced pressure. The residue was diluted with EtOAc and washed with saturated $NaHCO_3$ solution. The aqueous portion was extracted with fresh EtOAc, and the combined organic extracts were washed with brine, dried ($MgSO_4$), and evaporated under reduced pressure. The crude material (342 mg) was chromatographed on silica gel, eluting with methanol, to yield the purified product as a white solid (167 mg, 43%): IR ($CHCl_3$, cm^{-1}) 3024, 2975, 2934, 1593, 1456, 1378, 1314, 1167; FAB-MS *m/z* 1610 (M + H^+). Anal. Calcd ($C_{84}H_{143}N_3O_{26}$): C, 62.62; H, 8.95; N, 2.61. Found: C, 61.67; H, 8.73; N, 2.44.

RESULTS AND DISCUSSION

Structure of T-2. The structure of T-2, isolated and purified as previously reported ((Donoho *et al.*, 1992), was elucidated as compound 1 (Figure 1) by physical chemical methods. Its molecular weight was established as 1609 by FAB-mass spectrometry, with an elemental composition of $C_{84}H_{143}N_3O_{26}$. An initial examination of the ^1H and ^{13}C NMR spectra revealed the presence of two nonequivalent subunits derived from desmycosin plus one subunit from 3,5-dimethylpiperidine, indicating the combination of one moiety derived from tilmicosin (3) and one from desmycosin (4). Significantly, the NMR signal for a proton of an aldehyde group was not observed. Addition of the elemental compositions of tilmicosin ($C_{46}H_{80}N_2O_{13}$) and 20-dihydro-20-deoxydesmycosin ($C_{39}H_{67}NO_{13}$) yielded the elemental composition of $C_{85}H_{147}N_3O_{26}$, equivalent to T-2 plus CH_3, suggesting that T-2 may have arisen by loss of a methyl group and a hydrogen atom followed by coupling of the radicals thereby derived from the above two subunits. The NMR spectra clearly showed four *O*-methyl groups, indicating no change within the mycinosyl moieties. However, the *N*-methyl groups on mycaminose could not be accurately counted, thereby directing attention toward N-demethylation to potentially account for the missing methyl group. High-resolution measurements of the ions at *m/z* 933, 931, 915, 883, 867, 855, and 824 and analysis of their possible origins in the FAB-MS fragmentation pattern then led to the working model (1) that was proposed for the structure of T-2. Finally, the identification of T-1 as 3′-*N*-demethyltilmicosin (2) indicated that the loss of an *N*-methyl group was a reasonable hypothesis which could provide a self-consistent explanation for the results (*vide infra*).

Initial Synthesis of T-2. The structure of T-2 was proven to be that illustrated as compound 1 by synthesis of an authentic sample which was initially prepared from desmycosin following a well-precedented route (Figure 2). After protection of the aldehyde as its diethyl ketal (Tanaka *et al.*, 1981; Kirst *et al.*, 1988), N-demethylation was effected by iodine under basic conditions according to a process previously developed for erythromycin (Freiberg, 1973). N-Demethylation of spiramycin has also been reported by related procedures using *N*-bromosuccinimide as the source of a positive halogen agent (Sano *et al.*, 1985). Reductive amination of the secondary amino group in 6 with desmycosin under the usual conditions (Borch *et al.*, 1971) yielded the desired dimeric moiety, which was then converted to T-2 by acidic hydrolysis of the ketal followed by reductive amination of the liberated aldehyde group with 3,5-dimethylpiperidine under previously described conditions (Kirst *et al.*, 1989; Debono *et al.*, 1989). The synthetic material was identical to the isolated sample of T-2 in all respects, thereby firmly establishing its structure.

Initial Synthesis of T-1. A sample of 3′-*N*-demethyltilmicosin (T-1) was initially obtained from the key intermediate 6 by hydrolysis of the diethyl ketal and subsequent reductive amination of the aldehyde group with 3,5-dimethylpiperidine. The comparison of T-1 and tilmicosin by NMR indicated that the only points of difference were centered around the amino sugar, my-

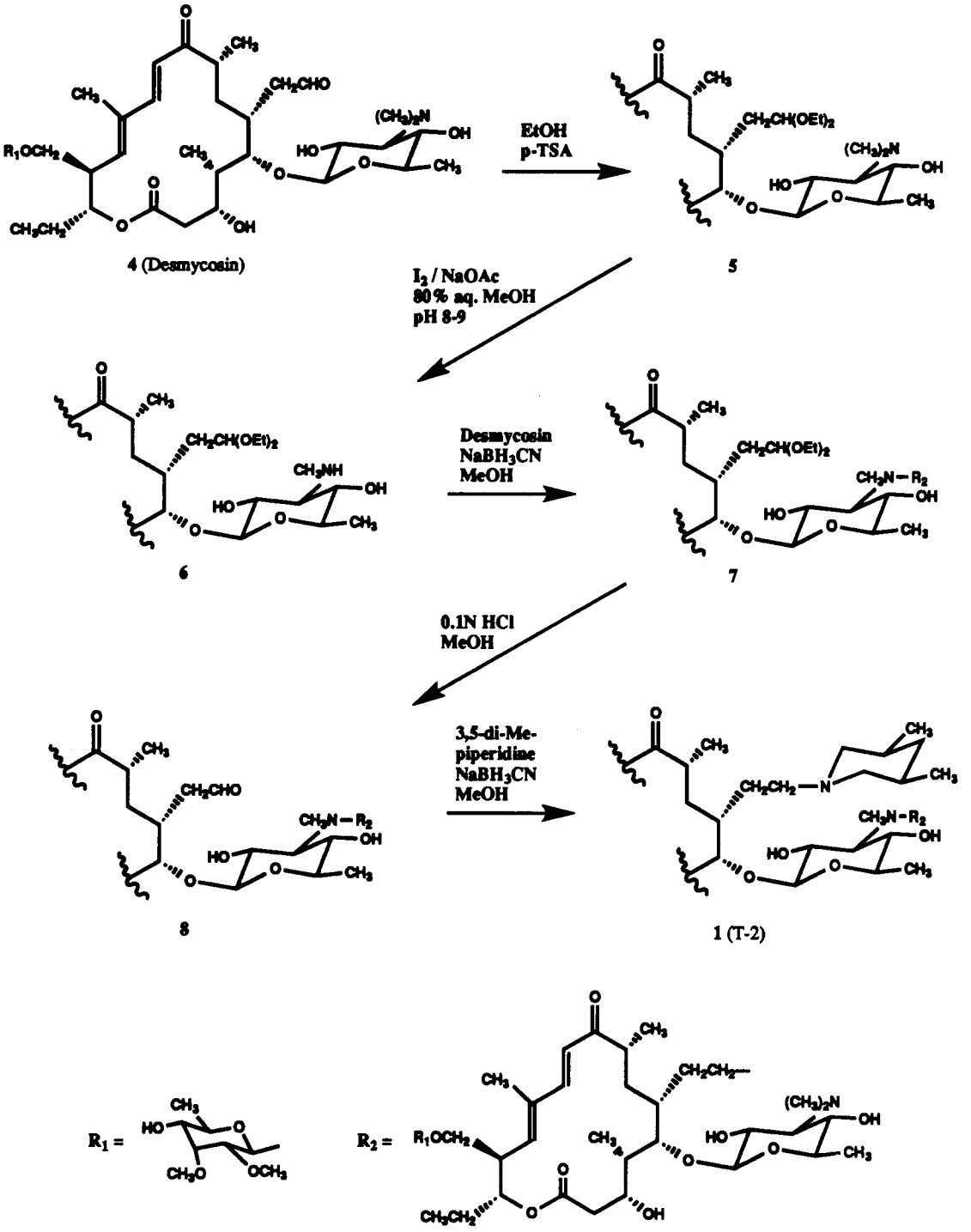


Figure 2. Initial synthesis of T-2.

caminose. The proton NMR spectra revealed only one *N*-methyl group present in T-1, in contrast to the dimethylamino group present in tilmicosin. The ^{13}C NMR spectra showed that the *N*-methyl and C-3′ resonances had shifted to lower values in T-1 due to the absence of a β effect, while C-2′ and C-4′ resonances shifted to higher values due to the absence of a γ effect (see Table 1). The other carbon resonances remained essentially unchanged.

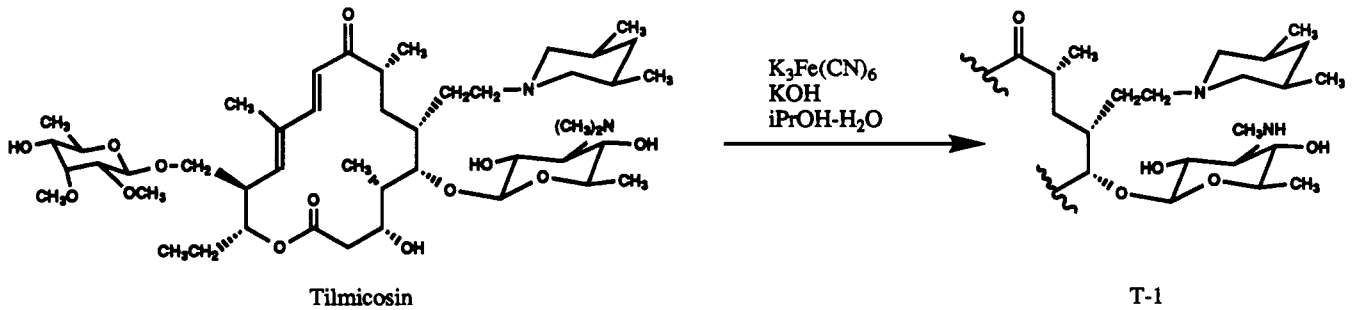


Figure 3. One-step synthesis of T-1.

Comparison of the synthetically derived material with the previously isolated metabolite showed that they were identical in all respects. However, the synthetic route from 6 to T-1 was not an efficient process, because dimerization of *N*-demethyldesmycosin with itself occurred under the reductive amination conditions. Consequently, a more efficient procedure was sought to conveniently provide gram quantities of material for further investigations.

Even though N-demethylation of compound 5 had been successfully achieved with iodine, all attempts to N-demethylate tilmicosin itself with positive halogen reagents were unsuccessful. Under milder conditions, no reaction occurred and starting material was recovered. Under more vigorous conditions, only complex mixtures were formed with no evidence of the desired product. The presence of the dimethylpiperidinyl substituent containing a more basic amino functionality apparently altered the reactivity of the molecule, leading in uncharacterized directions to undesired products.

Efficient Syntheses of T-1 and T-2. A thorough literature search uncovered a brief paper describing the use of $K_3Fe(CN)_6$ for N-demethylation of water-soluble secondary amino groups (Perrine, 1951). Although our initial attempts to use this procedure on tilmicosin were unsuccessful, the failure was presumed to be due to the insolubility of the substrate in such a highly basic solvent. Since secondary alcohols were reported to be compatible with the reagent, a mixed-solvent system of aqueous 2-propanol was therefore developed to solubilize tilmicosin, resulting in a successful and direct N-demethylation (Figure 3). This process was subsequently utilized to selectively N-demethylate tilmicosin on a 2-g scale in 42% yield (not optimized).

With T-1 readily available via a one-step synthesis from tilmicosin, reductive amination of the secondary amino group with desmycosin and $NaBH_3CN$ then directly yielded the dimeric compound T-2 in two steps from tilmicosin. Samples of both T-1 and T-2 produced by these more efficient synthetic routes were identical to the respective materials previously obtained, thereby making both compounds readily available for subsequent investigations.

LITERATURE CITED

Borch, R. F.; Bernstein, M. D.; Durst, H. D. The Cyanohydridoborate Anion as a Selective Reducing Agent. *J. Am. Chem. Soc.* **1971**, *93*, 2897–2904.

Debono, M.; Willard, K. E.; Kirst, H. A.; Wind, J. A.; Crouse, G. D.; Tao, E. V.; Vicenzi, J. T.; Counter, F. T.; Ott, J. L.; Ose, E. E.; Omura, S. Synthesis and Antimicrobial Evaluation of 20-Deoxo-20-(3,5-Dimethylpiperidin-1-yl)desmycosin (Tilmicosin, EL-870) and Related Cyclic Amino Derivatives. *J. Antibiot.* **1989**, *42*, 1253–1267.

Donoho, A. L.; Thomson, T. D.; Giera, D. D. Tilmicosin in Cattle: Metabolism and Tissue Residues. In *Xenobiotics and Food-Producing Animals: Metabolism and Residues*; Hutson, D. H., Hawkins, D. R., Paulson, G. D., Struble, C. B., Eds.; ACS Symposium Series 503; American Chemical Society: Washington, DC, 1992; pp 158–167.

Freiberg, L. A. Process for the Demethylation of 3-Amino Macrolides. U.S. Pat. 3,725,385, 1973.

Kirst, H. A.; Toth, J. E.; Debono, M.; Willard, K. W.; Truedell, B. A.; Ott, J. L.; Counter, F. T.; Felty-Duckworth, A. M.; Pekarek, R. S. Synthesis and Evaluation of Tylosin-Related Macrolides Modified at the Aldehyde Function: A New Series of Orally Effective Antibiotics. *J. Med. Chem.* **1988**, *31*, 1631–1641.

Kirst, H. A.; Willard, K. E.; Debono, M.; Toth, J. E.; Truedell, B. A.; Leeds, J. P.; Ott, J. L.; Felty-Duckworth, A. M.; Counter, F. T. Structure-Activity Studies of 20-Deoxo-20-Amino Derivatives of Tylosin-Related Macrolides. *J. Antibiot.* **1989**, *42*, 1673–1683.

Laven, R.; Andrews, A. H. Long-Acting Antibiotic Formulations in the Treatment of Calf Pneumonia: a Comparative Study of Tilmicosin and Oxytetracycline. *Vet. Rec.* **1991**, *129*, 109–111.

Morck, D. W.; Merrill, J. K.; Thorlakson, B. E.; Olson, M. E.; Tonkinson, L. V.; Costerton, J. W. Prophylactic Efficacy of Tilmicosin for Bovine Respiratory-Tract Disease. *J. Am. Vet. Med. Assoc.* **1993**, *202*, 273–277.

Ose, E. E. *In Vitro* Antibacterial Properties of EL-870, a New Semi-Synthetic Macrolide Antibiotic. *J. Antibiot.* **1987**, *40*, 190–194.

Perrine, T. D. N-Demethylation with Potassium Ferricyanide. *J. Org. Chem.* **1951**, *16*, 1303–1307.

Picavet, T.; Muylle, E.; Devriese, L. A.; Geryl, J. Efficacy of Tilmicosin in Treatment of Pulmonary Infections in Calves. *Vet. Rec.* **1991**, *129*, 400–403.

Sano, H.; Tanaka, H.; Yamashita, K.; Okachi, R.; Omura, S. Chemical Modification of Spiramycins. *J. Antibiot.* **1985**, *38*, 186–196.

Tanaka, A.; Watanabe, A.; Tsuchiya, T.; Umezawa, S.; Umezawa, H. Syntheses of 4′-Deoxy-Demycarosyl Tylosin and its Analogues. *J. Antibiot.* **1981**, *34*, 1381–1384.

Received for review November 15, 1993. Accepted February 23, 1994.[⊗]

[⊗] Abstract published in *Advance ACS Abstracts*, April 1, 1994.