SYNTHESIS AND STRUCTURE-ACTIVITY STUDIES OF NEW 4″-*O*-ACYLTYLOSIN DERIVATIVES OF THERAPEUTIC INTEREST†

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Eleven 4″-*O*-acyltylosin derivatives were synthesized and subjected to a two-step screening system consisting of antimicrobial activity and esterase stability assays. The new derivatives were all active against macrolide-resistant Staphylococci and mycoplasmas, but only 4″-*O*-(4-methoxy)phenylacetyltylosin and 4″-*O*-(4-acetyl)phenylacetyltylosin showed better resistance to mouse liver esterase than 4″-*O*-phenylacetyltylosin (reference compound **C**).

As a line of approach to overcoming the increasing frequency of occurrence of macrolide-resistant pathogens among recent clinical isolates, TSUCHIYA *et al.* synthesized a variety of tylosin derivatives, among which 4″-*O*-phenylthioacetyltylosin (reference compound **A**) was the most active *in vitro* against macrolide-resistant microorganisms.[2~4] In spite of its significantly improved antimicrobial activity *in vitro*, however, reference compound **A** yielded poor therapeutic effects on mouse infections experimentally caused by macrolide-resistant pathogens [MIC and ED_{50} data: Against *Staphylococcus aureus* Smith (a macrolide-sensitive strain) 0.8 μg/ml, 140 mg/kg (po) and 9 mg/kg (sc) for reference compound **A**, and 1.6 μg/ml and 110 mg/kg (po) for tylosin; against an erythromycin+oleandomycin+josamycin +tylosin-resistant strain of *S. aureus* (a clinical isolate) 12.5 μg/ml and >400 mg/kg (po and sc) for reference compound **A**, and >500 μg/ml and >400 mg/kg (po and sc) for tylosin (unpublished work)].[5] A major reason for no *in vivo* activity of reference compound **A** was found in the rapid removal of the 4″-*O*-phenylthioacetyl group by hepatic esterase, giving tylosin which was inactive against macrolide-resistant pathogens.[3] 3-*O*-Acetyl-4″-*O*-(3-pyridyl)acetyltylosin (reference compound **B**) and 4″-*O*-phenylacetyltylosin (reference compound **C**), although slightly less antimicrobial, were found to be more stable to hepatic esterase than reference compound **A**. Based on these findings, the previous evaluation system was revised to construct a two-step screening system comprising the antimicrobial activity assay and the hepatic esterase stability assay.

The present paper describes the synthesis and chemical structure-activity relationship of 11 new 4″-*O*-acyltylosin derivatives. A new method for 4″-*O*-acylation of tylosin is also presented.

Results and Discussion

New Method for 4″-*O*-Acylation of Tylosin

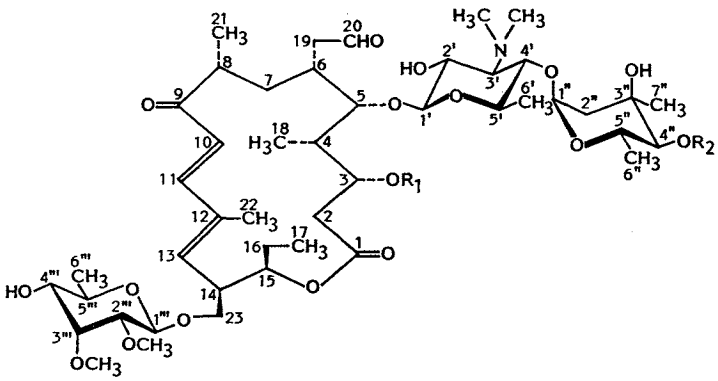
Tylosin, a 16-membered macrolide antibiotic, is composed of tylonolide, mycaminose, mycarose

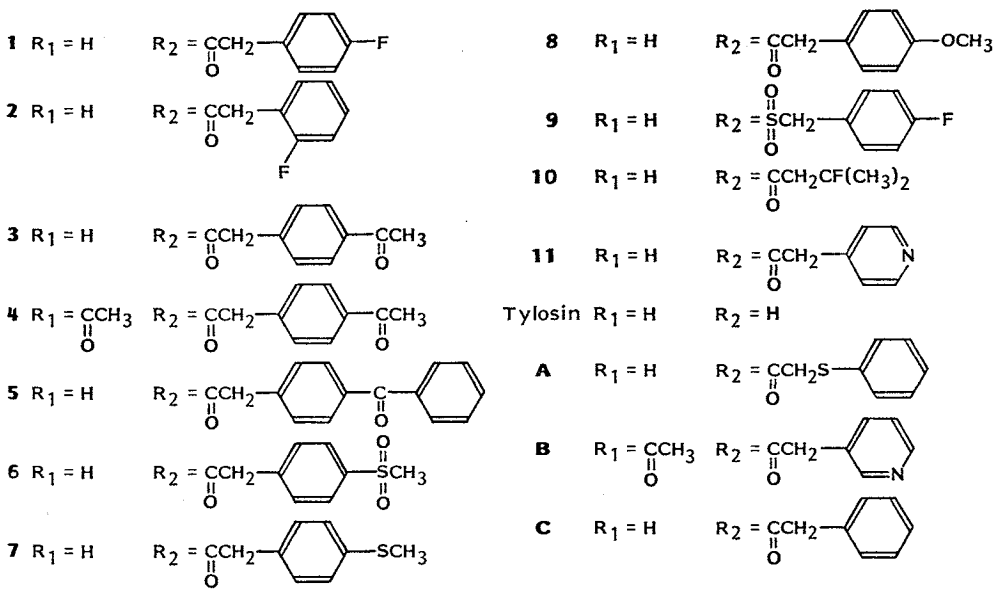
† See ref 1.

and mycinose (Fig. 1), and has four secondary and one tertiary hydroxyl groups. Among the four secondary hydroxyls, the chemical reactivity for acylation falls in the decreasing order of 2′, 4‴, 4″ and 3. The 2′-hydroxyl is readily acylable with an acid anhydride at room temperature even in the absence of a base by virtue of the neighboring effect of the dimethylamino group, while the 3-hydroxyl is least acylated at a low temperature as low as $-5\sim+8$°C, presumably because of the hydrogen bond formation with the lactone carbonyl.[6] Accordingly, the strategy of efficient 4″-*O*-acylation depends on how sophisticatedly the delicate difference in reactivity between the 4″- and 4‴-hydroxyl groups is controlled over the course of acylation and deprotection under conditions where the 3-hydroxyl is not acylated.

TSUCHIYA *et al.*[2] employed a preparation process (Method I; in Scheme 1) which consisted of protecting the 4‴-hydroxyl of 2′-*O*-acetyltylosin with a suitable group such as chloroacetyl; and then introducing a desired acyl group at 4″, followed by alcoholytic deprotection of the 2′-*O*-acetyl and 4‴-*O*-chloroacetyl groups. In this process, the 4‴-*O*-protection was completed as rapidly as in 5

Fig. 1. Structures of new 4″-*O*-acyltylosin derivatives.





Scheme 1.

Method I (TSUCHIYA *et al.*[2])

Tylosin
↓ 96% yield
2′-*O*-Acetyltylosin
↓ 60% yield
2′-*O*-Acetyl-4‴-*O*-chloroacetyltylosin
↓ 67% yield
2′-*O*-Acetyl-4‴-*O*-chloroacetyl-4″-*O*-phenylthioacetyltylosin
↓ 72% yield
4″-*O*-Phenylthioacetyltylosin (overall yield, 28%)

Method II (this paper)

Tylosin
↓ 96% yield
2′-*O*-Acetyltylosin
↓ 72% yield
2′-*O*-Acetyl-4″,4‴-di-*O*-phenylthioacetyltylosin
↓ 100% yield
4″,4‴-Di-*O*-phenylthioacetyltylosin
↓ 73% yield
4″-*O*-Phenylthioacetyltylosin (overall yield, 50%)

minutes at $-10\sim-15$°C, which made it very difficult to carry out a large-scale preparation of 4″-*O*-acyltylosin derivatives in good yields.

The above-described defect of Method I was solved by a new method (Method II; in Scheme 1) in this paper. After 2′-*O*-acetylation, the 4″- and 4‴-hydroxyls of tylosin were acylated with the same acyl group, and then only the 2′-*O*-acetyl group was removed by methanolysis. Finally the 4‴-*O*-acyl was selectively deprotected by treatment with ammonia in methanol. Except for derivatives **8** and **9** (Fig. 1), the overall yields of 4″-*O*-acyltylosins by Method II were $2\sim3$ times better than those by Method I, as the new method allowed the more selective acylation of the 4″- and 4‴-hydroxyls under milder deacylation conditions. As an example, the observed reaction yields of Methods I and II are comparatively shown at each step for 4″-*O*-phenylthioacetyltylosin (reference compound **A**) in Scheme 1.

Chemical structures of 4″-*O*-acyltylosins and related intermediates were confirmed by spectrometry. In general, with reference to tylosin and 3-*O*-acetyltylosin, the new 4″-*O*-acyltylosin derivatives in Fig. 1 showed the following common characteristics: The lowfield shifts of 4″-H by $1.6\sim1.7$ ppm and of C-4″ by $1.9\sim2.5$ ppm, and the highfield shift of C-5″ by $2.4\sim2.9$ ppm in the ^{1}H and ^{13}C NMR spectra.[6~8]

Antimicrobial and Enzymatic Evaluations of 4″-*O*-Acyltylosin Derivatives and Structure-activity Relationship

Revised Screening System

As 4″-*O*-phenylthioacetyltylosin and previous tylosin derivatives were found unstable *in vivo*, the screening system of TSUCHIYA[5] which was composed of the antibacterial assay with non-systematic esterase assay was revised to result in a new two-step assay system consisting of the antimicrobial screen and the esterase screen as follows:

1) The first-step screen was more expanded than the screening system of TSUCHIYA *et al.*[2] More particularly, tylosin derivatives were subjected to antimicrobial assays using standard and macrolide-resistant strains of bacteria and mycoplasmas as detector microbes; and 4″-*O*-phenylthioacetyltylosin and 4″-*O*-phenylacetyltylosin as reference macrolide compounds **A** and **C**, respectively.

2) The second-step screen examined the susceptibility to mouse liver esterase, as this enzyme was found to be the most responsible for deacylation of previous acylated tylosin derivatives, yielding tylosin which was inactive against macrolide-resistant pathogens. The reference compound in this test was

3-*O*-acetyl-4″-*O*-(3-pyridyl)acetyltylosin (reference compound **B**).

After several vain attempts such as 3″-*O*-acylation for stabilization of 4″-*O*-phenylthioacetyltylosin (reference compound **A**) to esterase, the authors decided to concentrate largely on a new series of 4″-*O*-phenylacetyltylosin derivatives, as 4″-*O*-phenylacetyltylosin (reference compound **C**) is relatively stable to hepatic esterase, but antimicrobially less active than reference compound **A**, whereas 4″-*O*-(4-nitro)phenylacetyltylosin was antimicrobially as active as 4″-*O*-phenylthioacetyltylosin (reference compound **A**), but unstable to hepatic esterase, suggesting that the introduction of a suitable substituent on the phenyl nucleus might give birth to a clinically useful tylosin derivative.

Structure-activity Relationship

1) Antimicrobial Activity:

1-a) Antibacterial Activity: Table 1 summarizes the comparative antibacterial activities of tylosin (parent compound), 4″-*O*-phenylthioacetyltylosin (reference compound **A**), 3-*O*-acetyl-4″-*O*-(3-pyridyl)acetyltylosin (reference compound **B**), 4″-*O*-phenylacetyltylosin (reference compound **C**), eleven new 4″-*O*-acyltylosin derivatives, erythromycin and josamycin.

As reported by TSUCHIYA *et al.*,[9] the introduction of a variety of acyl groups at the 4″-hydroxyl of tylosin (*e.g.* reference compounds **A**, **B** and **C**) gives tylosin derivatives that are active against three types of macrolide-resistant strains (type C which is resistant to erythromycin; type B which is resistant to erythromycin and oleandomycin; and type A which is resistant to all macrolide compounds).[10] It seems, however, that the introduction of aromatic acyl groups at 4″-OH of tylosin results in slight reduction in antibacterial potency, partly because of the molecular weight increase. In this paper, eight 4″-*O*-(substituted)phenylacetyltylosin derivatives (derivatives **1**~**8**) and three 4″-*O*-acyltylosin compounds (derivatives **9**~**11**) were synthesized.

Antibacterial comparison of 4″-*O*-phenylacetyltylosin (reference compound **C**) and derivatives **1** and **2** shows that fluorination increases the antibacterial potency of 4″-*O*-phenylacetyltylosin more at the *p*-position of the phenyl nucleus (derivative **1**) than at the *o*-position (derivative **2**). Accordingly the position of substituent introduction was set at the *p*-position of the phenyl nucleus.

p-Acetylation (derivative **3**) improves the antibacterial activity of reference compound **C**, but additional acetylation at 3-OH of tylosin (derivative **4**) has a negative influence on the favorable effect of the *p*-acetyl group in the phenylacetyl substituent. Benzoyl and methylsulfonyl groups (derivatives **5** and **6**) seem ineffective to improve the antibacterial activity of reference compound **C**. Surprisingly, like the electron-withdrawing nitro and acetyl groups, the electron-donating methylthio and methoxyl groups in derivatives **7** and **8**, respectively, have a favorable effect on antimicrobial activity.

4″-*O*-(*p*-Fluorophenyl)methylsulfonyltylosin (derivative **9**), 4″-*O*-(β-fluoro)isovaleryltylosin (derivative **10**) and 4″-*O*-(3-pyridyl)acetyltylosin (derivative **11**) have antibacterial activities similar or inferior to reference compound **C**. It is interesting to note that 3-*O*-acetyl-4″-*O*-(4-pyridyl)acetyltylosin (reference compound **B**) is less active than derivative **11** against the macrolide-resistant Staphylococci.

In conclusion, the antibacterial test revealed that derivatives **1**, **3**, **7** and **8** should be compared as promising primary candidates by the subsequent antimycoplasmal assay and esterase stability test.

1-b) Antimycoplasmal Activity: The antimycoplasmal activities of the reference compounds, the new tylosin derivatives, erythromycin and josamycin are compared in Table 2.

It is noteworthy that erythromycin and josamycin have no useful activity against the macrolide-

Table 1. Antibacterial activity of new 4″-*O*-acyltylosin derivatives and related macrolides (MIC in μg/ml).

Bacterium	Reference compound				4″-*O*-Substituted phenylacetyltylosin								4″-*O*-Acyltylosin			Control	
	Tylosin	A	B	C	1	2	3	4	5	6	7	8	9	10	11	EM	JM
Gram-positive bacteria																	
(a) Macrolide-resistant strains																	
Staphylococcus aureus EMf	0.78	1.56	1.56	1.56	1.56	1.56	0.78	1.56	1.56	1.56	1.56	0.39	1.56	1.56	1.56	>100	1.56
S. aureus MS 8710	>100	25	50	12.5	12.5	50	12.5	50	12.5	25	12.5	6.25	50	25	50	>100	>100
S. aureus MS 9351	>100	6.25	12.5	12.5	6.25	6.25	6.25	12.5	6.25	12.5	6.25	6.25	12.5	25	6.25	>100	>100
S. aureus MS 9610	>100	6.25	25	25	6.25	12.5	6.25	12.5	6.25	12.5	6.25	6.25	12.5	25	12.5	>100	>100
S. aureus MS 9861	3.12	3.12	3.12	3.12	1.56	3.12	1.56	3.12	3.12	0.78	3.12	3.12	1.56	3.12	3.12	>100	3.12
S. aureus MS 9937	25	3.12	6.25	3.12	1.56	3.12	3.12	3.12	6.25	3.12	3.12	3.12	3.12	3.12	3.12	12.5	>100
S. aureus MS 10225	1.56	1.56	3.12	3.12	0.78	1.56	0.78	1.56	3.12	0.78	1.56	1.56	1.56	1.56	1.56	>100	1.56
S. aureus MS 10246	>100	12.5	25	25	6.25	25	12.5	25	12.5	12.5	12.5	12.5	25	12.5	12.5	>100	>100
(b) Macrolide-sensitive strains																	
S. aureus 193	0.78	0.78	0.78	1.56	1.56	1.56	0.78	1.56	1.56	1.56	1.56	0.78	0.78	1.56	1.56	<0.20	0.39
S. aureus Smith	1.56	1.56	3.12	3.12	1.56	3.12	3.12	3.12	3.12	1.56	3.12	3.12	1.56	3.12	3.12	0.39	1.56
S. aureus FDA 209P	0.78	1.56	0.78	1.56	0.78	0.78	0.39	0.78	1.56	1.56	1.56	0.78	0.78	0.78	0.78	<0.20	0.39
Micrococcus luteus PCI 1001	<0.20	<0.20	<0.20	0.39	<0.20	<0.20	<0.20	<0.20	0.78	0.39	0.39	0.39	<0.20	<0.20	<0.20	<0.20	<0.20
Bacillus subtilis NRRL B-558	0.78	1.56	0.78	1.56	0.78	1.56	0.78	1.56	3.12	1.56	3.12	1.56	0.78	0.78	0.78	<0.20	0.78
Corynebacterium bovis 1810	<0.20	0.39	<0.20	0.39	<0.20	0.20	0.39	0.39	0.78	0.39	0.39	0.39	<0.20	<0.20	<0.20	<0.20	0.39
Gram-negative bacteria																	
Escherichia coli NIHJ	100	50	100	50	50	50	100	100	>100	100	100	50	50	100	50	6.25	100

EM: Erythromycin, JM: josamycin.

Table 2. Antimycoplasmal activity of new 4″-*O*-acyltylosin derivatives and related macrolides (MIC in μg/ml).

Mycoplasma	Reference compound				4″-*O*-Substituted phenylacetyltylosin								4″-*O*-Acyltylosin			Control	
	Tylosin	A	B	C	1	2	3	4	5	6	7	8	9	10	11	EM	JM
Macrolide-resistant strains																	
Mycoplasma gallisepticum S4A	0.20	<0.0002	0.012	0.024	0.006	0.024	0.006	0.012	0.012	0.006	0.024	0.003	0.012	0.024	0.006	100	6.25
M. gallisepticum A69	1.56	0.003	0.05	0.05	0.012	0.024	0.012	0.05	0.024	0.012	0.024	0.012	0.012	0.05	0.012	100	1.56
M. gallisepticum A72	0.78	0.0016	0.012	0.003	0.012	0.024	0.003	0.024	0.05	0.006	0.024	0.024	0.003	0.024	0.012	100	6.25
M. gallisepticum E7	0.39	0.0016	0.003	0.003	0.012	0.024	0.003	0.024	0.10	0.006	0.024	0.024	0.0016	0.024	0.012	25	12.5
M. gallisepticum E11	0.78	0.0016	0.05	0.003	0.012	0.024	0.012	0.05	0.024	0.006	0.024	0.012	0.024	0.05	0.012	100	25
M. gallisepticum E57	0.78	<0.0002	0.003	0.006	0.012	0.012	0.006	0.024	0.003	0.0016	0.024	0.0016	0.006	0.012	0.0016	>100	50
M. gallisepticum E103	0.39	0.003	0.012	0.003	0.012	0.024	0.006	0.024	0.10	0.012	0.05	0.05	0.0016	0.024	0.006	50	12.5
M. gallisepticum E112	1.56	0.003	0.05	0.003	0.012	0.006	0.012	0.05	0.05	0.012	0.024	0.012	0.012	0.05	0.024	25	6.25
M. pulmonis PG22	0.20	0.006	0.05	0.39	0.39	0.20	0.20	0.10	0.20	0.20	0.20	0.10	0.10	0.10	0.10	>100	25
Macrolide-sensitive strains																	
M. gallisepticum S6	0.10	0.003	0.10	0.024	0.024	0.006	0.012	0.006	0.05	0.012	0.024	0.012	0.006	0.024	0.024	0.024	0.10
M. gallisepticum TS18	0.0004	<0.0002	<0.0002	0.001	0.0004	<0.0001	<0.0002	0.0004	0.0008	<0.0002	0.0004	0.0016	<0.0002	<0.0001	<0.0002	0.012	0.024
M. pneumoniae Mac	0.0004	<0.0002	0.003	0.003	0.05	0.05	0.024	0.024	0.05	0.024	0.05	0.012	0.006	0.024	0.006	0.0008	0.05

EM: Erythromycin, JM: josamycin.

Table 3. Stability of new 4″-*O*-acyltylosin derivatives and related macrolides to hepatic esterase.

Incubation period (minutes)	Unchanged derivative (%) after incubation at 37°C													
	A	**B**	**C**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
30	0	91	91	89	85	96	96	83	87	90	97	100	—	84
60	0	87	85	68	75	96	92	75	75	87	93	100	83	81
120	0	78	68	26	44	91	90	69	68	72	91	100	—	77

resistant mycoplasmas, whereas tylosin itself exhibits a fairly good antimycoplasmal activity against such strains; and that all the 4″-*O*-acyl substituents produce markedly improved antimycoplasmal activities. As the MIC values are too small and the antimycoplasmal assay is more fluctuating than the antibacterial assay, it is least reliable to compare the antimycoplasmal activities of the reference macrolide compounds and new derivatives by their MIC values.

2) Esterase Stability: Table 3 shows the comparative susceptibilities of the reference and new tylosin derivatives to mouse liver esterase.

As reported by TSUCHIYA,[3] 4″-*O*-phenylthioacetyltylosin (reference compound **A**) is highly suceptible to esterase, whereas 3-*O*-acetyl-4″-*O*-(3-pyridyl)acetyltylosin (reference compound **B**) and 4″-*O*-phenylacetyltylosin (reference compound **C**) are relatively stable to the enzyme. It is unexpected to find that fluorination on the phenyl nucleus of the 4″-*O*-phenylacetyl substituent results in increased susceptibility to the esterase (derivatives **1** and **2**). Acetylation (derivatives **3** and **4**) significantly stabilizes 4″-*O*-phenylacetyltylosin (reference compound **C**) to hepatic esterase, and the presence of the 3-*O*-acetyl group (derivative **4**) changes no enzymatic susceptibility. The benzoyl and methanesulfonyl substituents (derivatives **5** and **6**) neither increase nor decrease the susceptibility to the enzyme. Like acetyl (derivative **3**), the methoxyl group is also effective to reduce the susceptibility to liver esterase (derivative **8**).

It is interesting to note that, although 4″-*O*-(β-hydroxy)isovaleryltylosin is known to be resistant to liver esterase,[3] 4″-*O*-(β-fluoro)isovaleryltylosin (derivative **10**) is relatively susceptible. 4″-*O*-(4-Pyridyl)acetyltylosin (derivative **11**) and 3-*O*-acetyl-4″-*O*-(3-pyridyl)acetyltylosin (reference compound **B**) are equal in their susceptibility to the esterase. The 4-fluorophenylmethylsulfonyl group gives tylosin complete resistance to the esterase, but is antibacterially unfavorable, as described above (derivative **9**).

In final conclusion, among the 4 primary candidates (derivatives **1**, **3**, **7** and **8**), 4″-*O*-(4-acetyl)-phenylacetyltylosin (derivative **3**) and 4″-*O*-(4-methoxy)phenylacetyltylosin (derivative **8**) passed the esterase screen and will comparatively be evaluated by animal tests.

Experimental

General Methods

MP's were determined on a Yanagimoto micro-mp apparatus and are uncorrected. ^{1}H and ^{13}C NMR spectra were recorded on a Varian EM-390 spectrometer and a Jeol GX-400 spectrometer, respectively. Chemical shifts are given in ppm from internal TMS in $CDCl_3$. UV and IR spectra were recorded with a Hitachi 200-20 UV/visible spectrophotometer and a Hitachi 260-30 IR spectrophotometer, respectively. Optical rotations were measured on a Jasco DIP-181. Field desorption MS data were obtained with a Hitachi RMU-7M mass spectrometer. Precoated Silica gel thin-layer plates F_{254} and Silica gel 60 (70~230 mesh) (E. Merck, Darmstadt) were employed for TLC and column chromatography, respectively.

4″-*O*-(4-Fluoro)phenylacetyltylosin (**1**) (Method II)

a) 2′-*O*-Acetyl-4″,4‴-di-*O*-(4-fluoro)phenylacetyltylosin

4-Fluorophenylacetic acid (5.0 g, 32 mmol) and triethylamine (4.5 ml, 32 mmol) were dissolved in 50 ml of methylene chloride and cooled to −15°C. Pivaloyl chloride (4.0 ml, 32 mmol) was added dropwise to the cool solution over 5 minutes under stirring, and agitation was continued for a further 15 minutes. Then 9 ml (110 mmol) of pyridine and 5.0 g (5.2 mmol) of 2′-*O*-acetyltylosin were mixed with the solution, which was stirred for 3 hours at 5°C. At the end of acylation, the reaction mixture was quenched with 20 ml of sodium bicarbonate solution and the organic layer was separated. After rinsing with 50 ml of sodium bicarbonate solution, followed by dehydration over anhydrous sodium sulfate, the solvent was removed by evaporation under reduced pressure. The evaporation residue was dissolved in 30 ml of toluene and evaporated to dryness *in vacuo* for complete removal of the pyridine. The crude preparation was dissolved in 15 ml of benzene and charged on a silica gel chromatographic column (150 g). Elution with a solvent mixture of benzene - acetone (7 : 1) gave fractions which showed a colored spot of Rf 0.47 on a silica gel TLC plate (developed in a solvent system of benzene - acetone (3 : 1); visualized with sulfuric acid). After the solvent was evaporated, the residue was washed with *n*-hexane to give 3.6 g of a white powder of 2′-*O*-acetyl-4″,4‴-di-*O*-(4-fluoro)phenylacetyltylosin (yield 56%): ^{1}H NMR ($CDCl_3$, major signals) δ 1.79 (3H, s, 22-H), 2.07 (3H, s, 2′-OAc), 2.40 (6H, s, $N(CH_3)_2$), 3.37 (3H, s, 2‴-OCH_3), 3.46 (3H, s, 3‴-OCH_3), 3.61 (2H, s, 4‴-$COCH_2$-Ar), 3.67 (2H, s, 4″-$COCH_2$Ar), 5.90 (1H, d, J=10 Hz, 13-H), 6.29 (1H, d, J=16 Hz, 10-H), 6.85～7.50 (9H, m, 11-H, 4″- and 4‴-Ar), 9.67 (1H, s, 20-H).

b) 4″-*O*-(4-Fluoro)phenylacetyltylosin (**1**)

2′-*O*-Acetyl-4″,4‴-di-*O*-(4-fluoro)phenylacetyltylosin (3.6 g) was refluxed in 100 ml of MeOH for 15 hours and then concentrated to 40 ml *in vacuo*. Under ice-cooling, the concentrate was mixed with 60 ml of 17% ammonia - MeOH and 8 ml of water, and agitated at 10°C for 7 hours. The reaction mixture was diluted with 25 ml of benzene and concentrated to 20 ml under reduced pressure. The concentrate was extracted with 100 ml of EtOAc and the aqueous layer was discarded. The organic layer was dried over anhydrous sodium sulfate and then evaporated to dryness *in vacuo*. Silica gel column (130 g) chromatography using a 3 : 1 mixture of benzene - acetone as eluant gave fractions which presented a colored spot of Rf 0.27 on a silica gel TLC plate (developed in a solvent system of benzene - acetone (3 : 2); visualized with sulfuric acid). Evaporation of the solvent from the combined fractions provided a white powder. The white powder was taken into 20 ml of benzene and the insoluble matters were removed by filtration. Forced precipitation with 150 ml of *n*-hexane gave 1.36 g of 4″-*O*-(4-fluoro)phenylacetyltylosin (**1**), yield 44%: MP 107～109°C; $[\alpha]_D^{24}$ −40.3° (*c* 1.0, MeOH); UV λ_{max}^{MeOH} nm (ε) 284 (20,000), 273 (sh), 267 (sh); IR ν_{max}^{KBr} cm^{-1} 1720, 1680, 1595; ^{1}H NMR ($CDCl_3$) δ 0.94 (3H, t, J=7 Hz, 17-H), 0.96 (3H, d, J=6 Hz, 6″-H), 1.01 (3H, d, J=7 Hz, 18-H), 1.09 (3H, s, 7″-H), 1.22 (3H, d, J=7 Hz, 21-H), 1.23 (3H, d, J=6 Hz, 6′-H), 1.26 (3H, d, J=6.5 Hz, 6‴-H), 1.42～1.70 (4H, m, 4-H, 7-H, 16-H), 1.79 (3H, s, 22-H), 1.82 (1H, dd, J=14 and 3.5 Hz, 2″-H_{ax}), 1.88 (1H, m, 16-H), 1.95 (1H, d, J=16.5 Hz, 2-H), 2.00 (1H, d, J=14 Hz, 2″-H_{eq}), 2.35～2.65 (4H, m, 6-H, 8-H, 19-H, 3′-H), 2.49 (6H, s, $N(CH_3)_2$), 2.88 (1H, dd, J=10 and 18 Hz, 19-H), 2.97 (1H, m, 14-H), 3.03 (1H, dd, J=3 and 8 Hz, 2‴-H), 3.18 (1H, m, 4‴-H), 3.23～3.30 (2H, m, 4′-H, 5′-H), 3.49 (3H, s, 2‴-OCH_3), 3.45～3.58 (3H, m, 23-H, 2′-H, 5‴-H), 3.62 (3H, s, 3‴-OCH_3), 3.69 (2H, ABq, J=15 Hz, CH_2-Ar), 3.71 (1H, d, J=10 Hz, 5-H), 3.75 (1H, t, J=3 Hz, 3‴-H), 3.83 (1H, d, J=10 Hz, 3-H), 4.00 (1H, dd, J=4 and 10 Hz, 23-H), 4.22 (1H, d, J=7.5 Hz, 1′-H), 4.41 (1H, m, 5″-H), 4.56 (1H, d, J=8 Hz, 1‴-H), 4.57 (1H, d, J=11 Hz, 4‴-H), 4.99 (1H, dt, J=2.5 and 10 Hz, 15-H), 5.06 (1H, d, J=3.5 Hz, 1″-H), 5.86 (1H, d, J=10.5 Hz, 13-H), 6.23 (1H, d, J=15.5 Hz, 10-H), 7.01 (2H, t, J=9 Hz, 3⁗-H, 5⁗-H), 7.29 (2H, dd, J=6 and 9 Hz, 2⁗-H, 6⁗-H), 9.67 (1H, s, 20-H); ^{13}C NMR ($CDCl_3$) δ 9.1 (C-18), 9.7 (C-17), 13.0 (C-22), 17.4 (C-21), 17.6 (C-6″), 17.8 (C-6‴), 18.9 (C-6′), 25.3 (C-7″), 25.5 (C-16), 31.8 (C-6), 32.8 (C-7), 39.4 (C-2), 40.3 (C-4), 40.5 (CH_2-Ar), 41.6 (C-2″), 41.9 ($N(CH_3)_2$), 43.8 (C-19), 44.7 (C-8), 45.1 (C-14), 59.7 (2‴-OCH_3), 61.8 (3‴-OCH_3), 63.3 (C-5″), 67.8 (C-3), 68.7 (C-3′), 69.1 (C-23), 69.3 (C-3″), 70.6 (C-5‴), 71.7 (C-2′), 72.7 (C-4‴), 73.1 (C-5′), 75.2 (C-15), 75.7 (C-4′), 77.8 (C-4″), 79.8 (C-3‴), 81.3 (C-5), 81.9 (C-2‴), 96.9 (C-1″), 101.1 (C-1‴), 103.7 (C-1′),

115.5 and 115.7 (C-3'''' and C-5''''), 118.7 (C-10), 129.6 (C-1''''), 130.9 and 131.0 (C-2'''' and C-6''''), 134.9 (C-12), 142.3 (C-13), 148.1 (C-11), 162.1 (C-4''''), 171.2 ($COCH_2$-Ar), 174.0 (C-1), 202.9 (C-20), 203.1 (C-9); secondary ion mass spectrum (SI-MS) *m/z* 1,052 (M+1).

4''-*O*-(2-Fluoro)phenylacetyltylosin (**2**) (Method I)

a) 2'-*O*-Acetyl-4'''-*O*-chloroacetyl-4''-*O*-(2-fluoro)phenylacetyltylosin

To a solution of 1.0 g (6.5 mmol) of (2-fluoro)phenylacetic acid in 15 ml of methylene chloride and 0.9 ml (6.5 mmol) of triethylamine was added 0.8 ml (6.5 mmol) of pivaloyl chloride at −15°C and the solution was stirred for 20 minutes at −15°C. Then 1.8 ml of pyridine and 1.0 g (1.96 mmol) of 2'-*O*-acetyl-4'''-*O*-chloroacetyltylosin[2] were added to the reaction mixture at 10°C and allowed to react for 8 hours under stirring. The resulting mixture was poured into an aqueous sodium bicarbonate solution and stirred. The organic layer was diluted with methylene chloride, rinsed with a saturated aqueous sodium bicarbonate solution and brine, and dried over anhydrous sodium sulfate. After the solvent was evaporated *in vacuo*, the residue was taken into 30 ml of toluene and subjected to evaporation *in vacuo* so that the pyridine was completely removed. This step might be necessary to repeat several times.

The crude product was dissolved in a small volume of benzene and charged on a silica gel chromatographic column (30 g). The column was developed successively with benzene - acetone mixtures (10 : 1, 7 : 1 and 6 : 1). Eluate fractions which showed a UV-absorbing spot of Rf 0.59 on a silica gel TLC plate (developed with a 2 : 1 mixture of benzene - acetone) were combined and concentrated to dryness *in vacuo* to give 810 mg (yield 72%) of 2'-*O*-acetyl-4'''-*O*-chloroacetyl-4''-*O*-(2-fluoro)phenylacetyltylosin; ^{1}H NMR ($CDCl_3$, major signals only) δ 1.80 (3H, s, 22-H), 2.08 (3H, s, 2'-OAc), 2.40 (6H, s, $N(CH_3)_2$), 3.50 (3H, s, 2'''-OCH_3), 3.53 (3H, s, 3'''-OCH_3), 3.76 (2H, s, 4''-OCOCH_2-Ar), 4.08 (2H, s, 4'''-$OCOCH_2Cl$), 5.90 (1H, d, $J_{13,14}$=10.5 Hz, 13-H), 6.28 (1H, d, $J_{10,11}$=15.5 Hz, 10-H), 6.90~7.45 (5H, m, 11-H, Ar-H), 9.68 (1H, s, 20-H).

b) 4''-*O*-(2-Fluoro)phenylacetyltylosin (**2**)

2'-*O*-Acetyl-4'''-*O*-chloroacetyl-4''-*O*-(2-fluoro)phenylacetyltylosin (810 mg) was dissolved in 20 ml of MeOH and refluxed for 24 hours. After the solvent was evaporated, the residue in benzene was applied on a silica gel chromatographic column (30 g) and eluted with a 2 : 1 mixture of benzene and acetone. Fractions which gave a colored spot of Rf 0.27 (silica gel TLC in a 3 : 2 mixture of benzene - acetone; visualized with dil sulfuric acid) were collected and concentrated to dryness *in vacuo*, affording 460 mg (yield 58%) of 4''-*O*-(2-fluoro)phenylacetyltylosin (**2**): MP 118~120°C; $[\alpha]_D^{24}$ −44.3° (*c* 1.0, MeOH); UV λ_{max}^{MeOH} nm (ε) 283.5 (21,000), 270 (sh), 264 (sh); IR $\nu_{max}^{CHCl_3}$ cm^{-1} 1720, 1675, 1585; ^{1}H NMR ($CDCl_3$) δ 1.80 (3H, s, 22-H), 2.50 (6H, s, $N(CH_3)_2$), 3.49 (3H, s, 2'''-OCH_3), 3.61 (3H, s, 3'''-OCH_3), 3.77 (2H, s, OCOCH_2-Ar), 5.92 (1H, d, *J*=10 Hz, 13-H), 6.25 (1H, d, *J*=16 Hz, 10-H), 6.90~7.45 (5H, m, 11-H, Ar-H), 9.68 (1H, s, 20-H).

4''-*O*-(4-Acetyl)phenylacetyltylosin (**3**)

Method I gave a 40%-yield of **3** from 2'-*O*-acetyl-4'''-*O*-chloroacetyltylosin, whereas Method II resulted in 57%: Rf 0.46 (silica gel TLC, $CHCl_3$ - MeOH, 10 : 1); mp 116~119°C; $[\alpha]_D^{24}$ −35.2° (*c* 1.0, MeOH); UV λ_{max}^{MeOH} nm (ε) 283 (20,000), 254 (18,000); IR $\nu_{max}^{CHCl_3}$ cm^{-1} 1720, 1680, 1590; ^{1}H NMR ($CDCl_3$) δ 1.80 (3H, s, 22-H), 2.49 (6H, s, $N(CH_3)_2$), 2.58 (3H, s, Ar-*Ac*), 3.49 (3H, s, 2'''-OCH_3), 3.61 (3H, s, 3'''-OCH_3), 3.78 (2H, s, OCOCH_2-Ar), 5.92 (1H, d, *J*=10 Hz, 13-H), 6.24 (1H, d, *J*=16 Hz, 10-H), 7.32 (1H, d, *J*=16 Hz, 11-H), 7.43 (2H, d, *J*=8 Hz, Ar-H), 7.92 (2H, d, *J*=8 Hz, Ar-H), 9.69 (1H, s, 20-H).

3-*O*-Acetyl-4''-*O*-(4-acetyl)phenylacetyltylosin (**4**)

The yield of **4** from 3-*O*-acetyltylosin was 33% by Method II: Rf 0.27 (silica gel TLC, benzene - acetone, 3 : 1); mp 107~111°C; $[\alpha]_D^{24}$ −28.9° (*c* 1.0, MeOH); UV λ_{max}^{MeOH} nm (ε) 282.5 (22,000), 253.5 (20,000); IR ν_{max}^{KBr} cm^{-1} 1730, 1675, 1590; ^{1}H NMR ($CDCl_3$) δ 1.79 (3H, s, 22-H), 2.07 (3H, s, 3-OAc), 2.46 (6H, s, $N(CH_3)_2$), 2.54 (3H, s, Ar-*Ac*), 3.42 (3H, s, 2'''-OCH_3), 3.57 (3H, s, 3'''-OCH_3), 3.73 (2H, s, CH_2-Ar), 5.89 (1H, d, *J*=10 Hz, 13-H), 6.19 (1H, d, *J*=16 Hz, 10-H), 7.32 (1H, d, *J*=16 Hz, 11-H),

7.34 (2H, d, J=8 Hz, Ar-H), 7.84 (2H, d, J=8 Hz, Ar-H), 9.55 (1H, s, 20-H).

4″-*O*-(4-Benzoyl)phenylacetyltylosin (**5**)

2′-*O*-Acetyl-4‴-*O*-chloroacetyltylosin was led to **5** in 54% yield by Method I: Rf 0.26 (silica gel TLC, benzene - acetone, 3 : 2); mp 107.5~109.5°C; $[\alpha]_D^{24}$ −36.2° (c 1.0, MeOH); UV λ_{max}^{MeOH} nm (ε) 272 (22,000); IR ν_{max}^{KBr} cm^{-1} 1720, 1670, 1650 (benzoyl), 1590; ^{1}H NMR ($CDCl_3$) δ 1.78 (3H, s, 22-H), 2.46 (6H, s, $N(CH_3)_2$), 3.46 (3H, s, 2‴-OCH_3), 3.57 (3H, s, 3‴-OCH_3), 3.77 (2H, s, $OCOCH_2$-Ar), 5.85 (1H, d, J=10 Hz, 13-H), 6.20 (1H, d, J=16 Hz, 10-H), 7.10~7.80 (10H, m, 11-H, Ar-H), 9.60 (1H, s, 20-H).

4″-*O*-(4-Methylsulfonyl)phenylacetyltylosin (**6**)

The yield of **6** was found to be 57% from 2′-*O*-acetyl-4‴-*O*-chloroacetyltylosin by Method I: Rf 0.23 (silica gel TLC, benzene - acetone, 3 : 2); mp 121~126°C; $[\alpha]_D^{24}$ −40.7° (c 1.0, MeOH); UV λ_{max}^{MeOH} nm (ε) 283.5 (20,000), 223.5 (14,000); IR ν_{max}^{KBr} cm^{-1} 1720 (ester, aldehyde), 1675 (conjugated ketone), 1590 (double bond), 1305, 1145 (sulfone); ^{1}H NMR ($CDCl_3$) δ 1.76 (3H, s, 22-H), 2.47 (6H, s, $N(CH_3)_2$), 3.00 (3H, s, SO_2CH_3), 3.46 (3H, s, 2‴-OCH_3), 3.59 (3H, s, 3‴-OCH_3), 3.78 (2H, s, $OCOCH_2$-Ar), 5.85 (1H, d, J=10 Hz, 13-H), 6.18 (1H, J=16 Hz, 10-H), 7.23 (1H, d, J=16 Hz, 11-H), 7.45 (2H, d, J=8 Hz, Ar-H), 7.82 (2H, d, J=8 Hz, Ar-H), 9.60 (1H, s, 20-H).

4″-*O*-(4-Methylthio)phenylacetyltylosin (**7**)

Reaction yield by Method II was 45%: Rf 0.50 (silica gel TLC, benzene - acetone, 3 : 2); mp 105~108°C; $[\alpha]_D^{24}$ −35.1° (c 1.0, MeOH); UV λ_{max}^{MeOH} nm (ε) 281 (19,000), 262.5 (19,400); IR ν_{max}^{KBr} cm^{-1} 1720, 1675, 1590; ^{1}H NMR ($CDCl_3$, major peaks only) δ 1.77 (3H, s, 22-H), 2.41 (3H, s, Ar-SCH_3), 2.45 (6H, s, $N(CH_3)_2$), 3.45 (3H, s, 2‴-OCH_3), 3.58 (3H, s, 3‴-OCH_3), 3.62 (2H, s, $OCOCH_2$-Ar-SCH_3), 6.19 (1H, d, J=16 Hz, 10-H), 7.16 (4H, s, *Ar*-SCH_3), 7.23 (1H, d, J=16 Hz, 11-H), 9.59 (1H, s, 20-H).

4″-*O*-(4-Methoxy)phenylacetyltylosin (**8**)

Yields of Methods I and II were 24 and 15%, respectively: Rf 0.25 (silica gel TLC, benzene - acetone, 2 : 1); mp 238~240°C; $[\alpha]_D^{24}$ −43.6° (c 1.0, MeOH); UV λ_{max}^{MeOH} nm (ε) 283 (24,200), 226 (11,300); IR ν_{max}^{KBr} cm^{-1} 1725 (ester, aldehyde), 1675 (conjugated ketone), 1590 (double bond); ^{1}H NMR ($CDCl_3$) δ 0.94 (3H, t, J=7.2 Hz, 17-H), 0.96 (3H, d, J=6 Hz, 6″-H), 1.01 (3H, d, J=7 Hz, 18-H), 1.09 (3H, s, 7″-H), 1.21 (3H, d, J=7 Hz, 21-H), 1.23 (3H, d, J=6 Hz, 6′-H), 1.26 (3H, d, J=6 Hz, 6‴-H), 1.41~1.70 (4H, m, 4-H, 7-H, 16-H), 1.80 (3H, s, 22-H), 1.81 (1H, dd, J=3.5 and 14.5 Hz, 2″-H_{ax}), 1.88 (1H, m, 16-H), 1.94 (1H, d, J=16.5 Hz, 2-H), 1.99 (1H, d, J=14.5 Hz, 2″-H_{eq}), 2.34 (1H, d, J=11 Hz, 4‴-OH), 2.35~2.65 (5H, m, 2-H, 6-H, 8-H, 19-H, 3′-H), 2.49 (6H, s, $N(CH_3)_2$), 2.88 (1H, dd, J=10 and 18 Hz, 19-H), 2.97 (1H, m, 14-H), 3.03 (1H, dd, J=3 and 8 Hz, 2‴-H), 3.18 (1H, dt, J=3 and 11 Hz, 4‴-H), 3.23~3.30 (2H, m, 4′-H, 5′-H), 3.49 (3H, s, 2‴-OCH_3), 3.49~3.58 (3H, m, 23-H, 2′-H, 5‴-H), 3.62 (3H, s, 3‴-OCH_3), 3.65 (2H, ABq, J=15 Hz, CH_2-Ar), 3.72 (1H, d, J=9 Hz, 5-H), 3.75 (1H, t, J=3 Hz, 3‴-H), 3.79 (3H, s, Ar-OCH_3), 3.82 (1H, d, J=10.5 Hz, 3-H), 4.00 (1H, dd, J=4 and 9.5 Hz, 23-H), 4.22 (1H, d, J=7.5 Hz, 1′-H), 4.41 (1H, m, 5″-H), 4.57 (1H, d, J=8 Hz, 1‴-H), 4.58 (1H, d, J=10 Hz, 4″-H), 4.99 (1H, dt, J=2.5 and 10 Hz, 15-H), 5.06 (1H, d, J=3.5 Hz, 1″-H), 5.92 (1H, d, J=10.5 Hz, 13-H), 6.27 (2H, d, J=15.5 Hz, 10-H), 6.85 (2H, d, J=9 Hz, 3‴′-H, 5‴′-H), 7.24 (2H, d, J=9 Hz, 2‴′-H, 6‴′-H), 7.32 (1H, d, J=15.5 Hz, 11-H), 9.68 (1H, s, 20-H); ^{13}C NMR ($CDCl_3$) δ 9.0 (C-18), 9.6 (C-17), 13.0 (C-22), 17.3 (C-21), 17.5 (C-6″), 17.7 (C-6‴), 18.8 (C-6′), 25.3 (C-7″), 25.5 (C-16), 32.0 (C-6), 32.8 (C-7), 39.3 (C-2), 40.1 (C-4), 40.4 (CH_2-Ar), 41.6 (C-2″), 41.8 ($N(CH_3)_2$), 43.8 (C-19), 44.6 (C-8), 45.0 (C-14), 55.2 (Ar-OCH_3), 59.7 (2‴-OCH_3), 61.7 (3‴-CH_3), 63.3 (C-5″), 67.8 (C-3), 68.7 (C-3′), 69.0 (C-23), 69.3 (C-3″), 70.6 (C-5‴), 71.7 (C-2′), 72.7 (C-4‴), 73.1 (C-5′), 75.2 (C-15), 75.8 (C-4′), 77.6 (C-4″), 79.8 (C-3‴), 81.4 (C-5), 81.9 (C-2‴), 96.9 (C-1″), 101.0 (C-1‴), 103.7 (C-1′), 114.0 (C-3‴′, C-5‴′), 118.9 (C-10), 126.0 (C-1‴′), 130.3 (C-2‴′, C-6‴′), 134.8 (C-12), 142.2 (C-13), 148.0 (C-11), 158.7 (C-4‴′), 171.6 (CO-CH_2Ar), 173.9 (C-1), 202.7 (C-20), 202.9 (C-9); field desorption (FD)-MS 1,064 (M+1).

4″-*O*-(4-Fluoro)phenylmethylsulfonyltylosin (**9**)

2′-*O*-Acetyl-4‴-*O*-chloroacetyltylosin yielded **9** in 46% yield by Method I: Rf 0.29 (silica gel TLC,

benzene - acetone, 2:1); mp 122~124°C; $[\alpha]_D^{24}$ −27.5° (*c* 1.0, MeOH); UV λ_{max}^{MeOH} nm (ε) 283 (19,000), 271 (sh), 265 (sh); IR ν_{max}^{KBr} cm^{-1} 1720, 1680, 1595; ^1H NMR ($CDCl_3$) δ 1.80 (3H, s, 22-H), 2.49 (6H, s, $N(CH_3)_2$), 3.50 (3H, s, 2‴-OCH_3), 3.62 (3H, s, 3‴-OCH_3), 4.41 (2H, s, SO_2CH_2-Ar), 5.09 (1H, d, *J*= 10 Hz, 13-H), 6.25 (1H, d, *J*=16 Hz, 10-H), 7.04 (2H, t, *J*=9 Hz, Ar-H), 7.32 (1H, d, *J*=16 Hz, 11-H), 7.45 (2H, d, *J*=9 Hz, Ar-H), 9.69 (1H, s, 20-H).

4″-*O*-(3-Fluoro-3-methyl)butyryltylosin (**10**)

tert-Butyl acetate (5.3 ml, 40 mmol) was added at −78°C to a mixture of 25 ml of *n*-butyllithium (40 mmol) and 5.6 ml of diisopropylamine in 25 ml of *n*-hexane, and stirring was continued for 30 minutes. After the temperature of the reaction mixture was raised to 0°C, 3 ml (40 mmol) of acetone was added and stirring was continued for a further 20 minutes. The content was poured into 50 g of ice water and adjusted to pH 2.0 with conc HCl. The organic layer was separated and washed successively with brine, a saturated aqueous sodium bicarbonate solution and saline. The organic solution was dried over anhydrous sodium sulfate and then subjected to evaporation to dryness *in vacuo*. Distillation under reduced pressure yielded 2.6 g of *tert*-butyl-3-hydroxy-3-methylbutyrate: BP 68~71°C/12 mmHg; ^1H NMR ($CDCl_3$) δ 1.26 (6H, s, $(CH_3)_2$), 1.47 (9H, s, *tert*-Bu), 2.38 (2H, s, CH_2), 3.73 (1H, s, OH).

The ester (1.54 g, 8.85 mmol) was added at −78°C to 20 ml of dry methylene chloride containing 1.3 ml (10.6 mmol) of diethylaminosulfur trifluoride and then the solution temperature was gently raised to room temperature. After 30 minutes of swirling, the reaction mixture was poured into 30 g of ice water. The organic layer was recovered and then rinsed successively with a saturated aqueous sodium bicarbonate solution and brine. Drying over anhydrous sodium sulfate, followed by concentration *in vacuo*, gave 5 ml of the concentrate. A mixture of the concentrate with 4 ml of trifluoroacetic acid was stirred for 60 minutes at room temperature, and the trifluoroacetic acid was completely removed by repeated evaporation from benzene. The carboxylic acid was treated with 1.4 ml of thionyl chloride at 40°C for 60 minutes. Concentration and subsequent distillation yielded 614 mg of (3-fluoro-3-methyl)butyryl chloride: BP 65°C/80 mmHg; ^1H NMR ($CDCl_3$) δ 1.50 (6H, d, *J*=22 Hz, $(CH_3)_2$), 3.25 (2H, d, *J*=15 Hz, CH_2).

Treatment of 2′-*O*-acetyl-4‴-*O*-chloroacetyltylosin with the acid chloride in pyridine, followed by deprotection, resulted in **10** in 31% yield: IR $\nu_{max}^{CHCl_3}$ cm^{-1} 1715, 1675, 1590; ^1H NMR ($CDCl_3$) δ 1.53 (6H, d, *J*=22 Hz, $CF(CH_3)_2$), 1.80 (3H, s, 22-H), 2.50 (6H, s, $N(CH_3)_2$), 2.77 (2H, d, *J*=18 Hz, $CH_2CF(CH_3)_2$), 3.48 (3H, s, 2‴-OCH_3), 3.60 (3H, s, 3‴-OCH_3), 5.92 (1H, d, *J*=10 Hz, 13-H), 6.25 (1H, d, *J*=16 Hz, 10-H), 7.32 (1H, d, *J*=16 Hz, 11-H), 9.70 (1H, s, 20-H).

4″-*O*-(4-Pyridyl)acetyltylosin (**11**)

Method I produced **11** in 26.5% yield from 2′-*O*-acetyl-4‴-*O*-chloroacetyltylosin: Rf 0.47 (silica gel TLC, $CHCl_3$- MeOH, 10: 1); mp 122~124°C; $[\alpha]_D^{24}$ −44.0° (*c* 1.0, MeOH); UV λ_{max}^{MeOH} nm (ε) 283.5 (22,000); IR ν_{max}^{KBr} cm^{-1} 1770, 1670, 1585; ^1H NMR ($CDCl_3$) δ 1.80 (3H, s, 22-H), 2.49 (6H, s, $N(CH_3)_2$), 3.47 (3H, s, 2‴-OCH_3), 3.61 (3H, s, 3‴-OCH_3), 3.71 (2H, s, $OCOCH_2$-pyridine), 5.88 (1H, d, *J*=10 Hz, 13-H), 6.23 (1H, d, *J*=16 Hz, 10-H), 7.23 (2H, d, *J*=5 Hz, pyridine-H), 7.31 (1H, d, *J*=16 Hz, 11-H), 8.52 (2H, d, *J*=5 Hz, pyridine-H), 9.67 (1H, s, 20-H).

Antimicrobial Assays

Antibacterial and antimycoplasmal assays were carried out as described before.[5]

Esterase Stability Assay

Male *dd*Y mice (25~30 g) were killed by venesection under ether anesthesia, and livers were collected and homogenized in 5 volumes of 0.1 M phosphate buffer, pH 7.2. The homogenate was centrifuged at 3,000 rpm for 10 minutes at 0°C to give a crude hepatic esterase suspension. As some tylosin derivatives were possibly hardly water-soluble, 5 mg of a tylosin derivative was first dissolved in 1.0 ml of MeOH and then diluted with 9.0 ml of water to give a 500-μg/ml macrolide solution. Two ml of the hepatic esterase suspension, 2.0 ml of the macrolide solution and 1.0 ml of 0.1 M phosphate buffer, pH 7.2 were vortexed for 5 seconds. After 30, 60 and 120 minutes of incubation at 37°C, 1.0 ml each of the reaction mixture was collected, inactivated at 85°C for 3 minutes and rapidly cooled down to room temperature. The reaction mixtures (1 ml each) were adjusted to pH 9.0 with 1 N NaOH

and subjected to extraction with 1.0 ml of EtOAc under agitation for 120 seconds. The solvent and the aqueous layer were separated by centrifugation at 3,000 rpm for 10 minutes and the solvent layer was assayed for intact macrolide by TLC.

Twenty μl amounts of the organic layers were spotted on a silica gel TLC plate (precoated Silica gel TLC plate F_{254}; E. Merck, Darmstadt) and developed in a solvent system of $CHCl_3$ - MeOH - 28% ammonia (150 : 12 : 1). The amounts of intact macrolide remaining in reaction mixtures were read by UV spectrophotometry at 282 nm in a Shimadzu Dual-wavelength TLC Scanner CS-930 and are shown in percent amounts by the following equation:

$$\frac{\text{Amount of intact macrolide remaining} \times 100}{\text{Initial amount of macrolide used}}$$

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