PHYSICO-CHEMICAL PROPERTIES OF NEW ACYL DERIVATIVES OF TYLOSIN PRODUCED BY MICROBIAL TRANSFORMATION

ROKURO OKAMOTO, TSUMORU FUKUMOTO, HIDEO NOMURA, KOHKI KIYOSHIMA, KENJI NAKAMURA and AKIRA TAKAMATSU

Central Research Laboratories, Sanraku-Ocean Co., Ltd.,
9–1, Johnan 4 chome, Fujisawa, 251 Japan

HIROSHI NAGANAWA, TOMIO TAKEUCHI and HAMAO UMEZAWA

Institute of Microbial Chemistry,
Kamiosaki, Shinagawa-ku, Tokyo, 141 Japan

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By microbial transformation of tylosin (**I**), the following eight new acyl derivatives were obtained: 3-acetyltylosin (**II**), 3-propionyltylosin (**III**), 4″-butyryltylosin (**IV**), 4″-isovaleryltylosin (**V**), 3-acetyl-4″-butyryltylosin (**VI**), 3-acetyl-4″-isovaleryltylosin (**VII**), 3-propionyl-4″-butyryltylosin (**VIII**) and 3-propionyl-4″-isovaleryltylosin (**IX**).

It is well known that the degree of the antibacterial activity and the blood levels of 16-membered macrolide antibiotics are influenced by the 3- and 4″-acyl groups.[1]

As reported in a previous paper,[2] 3- and 4″-hydroxyl groups of leucomycins, 16-membered macrolide antibiotics, were acylated by the culture of actinomycetes including *Streptomyces thermotolerans* ATCC 11416.

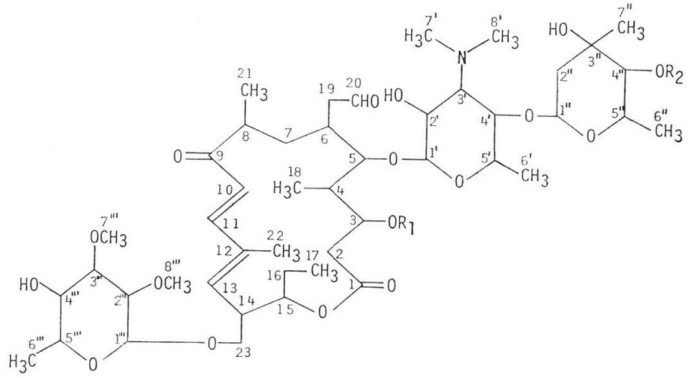
In a study to develop tylosin derivatives which produce a high blood level, we were succesful in the selective acylation of the 3- and 4″-hydroxyl groups using the culture of *S. thermotolerans* ATCC 11416 and obtained the following eight new tylosin derivatives: 3-acetyltylosin (**II**), 3-propionyltylosin (**III**), 4″-butyryltylosin (**IV**), 4″-isovaleryltylosin (**V**), 3-acetyl-4″-butyryltylosin (**VI**), 3-acetyl-4″-isovaleryltylosin (**VII**), 3-propionyl-4″-butyryltylosin (**VIII**) and 3-propionyl-4″-isovaleryltylosin (**IX**) (Fig. 1). This paper deals with the physico-chemical properties and the structure determination of these new acylated tylosin derivatives produced by microbial transformation. Their biological properties will be reported in a subsequent paper and the microbial acylation of tylosin and other macrolides will be detailed elsewhere.

Physico-chemical Properties

Similar to tylosin (**I**)[3,4] and other basic 16-membered macrolide antibiotics, all tylosin derivatives (**II**～**IX**) obtained by microbial transformation are very soluble in chloroform, acetone, ethyl acetate, benzene, lower alcohols and acidic water; and insoluble in petroleum ether and neutral water. They are crystallized as white platelets from ethyl ether or a mixture of ethyl ether and isopropyl ether (5: 2). They give positive anthrone, MOLISCH and sulfuric acid reactions and negative ninhydrin, biuret and EHRLICH reactions. They are stable and show no decomposition for 3 months at 80°C.

Melting point, optical rotation, UV absorption coefficient and Rf values on silica-gel plates are listed

Fig. 1. Structures and numbering of carbon atoms in tylosin acyl derivatives.



Compound		R_1	R_2
3-Acetyltylosin	(II)	$COCH_3$	H
3-Propionyltylosin	(III)	$COCH_2CH_3$	H
4″-Butyryltylosin	(IV)	H	$COCH_2CH_2CH_3$
4″-Isovaleryltylosin	(V)	H	$COCH_2CH(CH_3)_2$
3-Acetyl-4″-butyryltylosin	(VI)	$COCH_3$	$COCH_2CH_2CH_3$
3-Acetyl-4″-isovaleryltylosin	(VII)	$COCH_3$	$COCH_2CH(CH_3)_2$
3-Propionyl-4″-butyryltylosin	(VIII)	$COCH_2CH_3$	$COCH_2CH_2CH_3$
3-Propionyl-4″-isovaleryltylosin	(IX)	$COCH_2CH_3$	$COCH_2CH(CH_3)_2$
Tylosin	(I)	H	H

in Table 1. The common UV absorption maximum at 282 nm indicates the preservation of tylosin chromophore in all acyl derivatives.

Fig. 2 shows the IR absorption spectrum of **VII**. Although the IR absorption spectra of eight derivatives (**II**~**IX**) and tylosin (**I**) closely resemble with each other, the presence of the acetyl group in

Fig. 2. IR spectrum of 3-acetyl-4″-isovaleryltylosin (**VII**) (KBr).

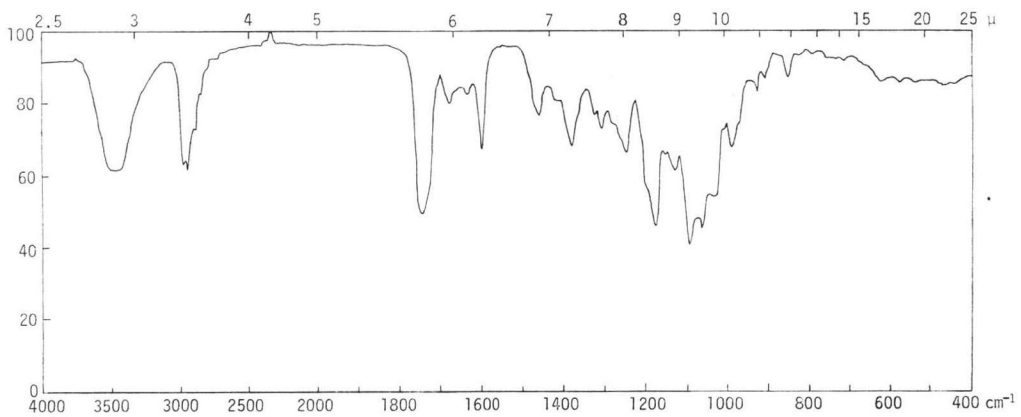


Table 1. Physico-chemical properties of acylated tylosin derivatives.

Compound	Melting point (°C)	$[\alpha]_D^{24}$ (c 1.0, MeOH)	λ_{max}^{EtOH} ($E_{1cm}^{1\%}$)	Rf values	
				A*	B*
II	213 ~216	−33.8	282 (237)	0.17	0.31
III	187 ~191.5	−32.1	282 (236)	0.21	0.34
IV	147 ~151	−53.4	282 (214)	0.45	0.66
V	154.5~156	−50.8	282 (217)	0.50	0.69
VI	178.5~181	−31.0	282 (240)	0.49	0.70
VII	147 ~151	−34.3	282 (222)	0.54	0.71
VIII	182 ~188	−35.2	282 (210)	0.54	0.72
IX	179 ~183	−33.1	282 (227)	0.59	0.74

Solvent system A*: *n*-hexane - acetone - methanol - benzene - ethyl acetate (30 : 10 : 8 : 25 : 20 by vol.)
B*: benzene - acetone (1 : 2 by vol.)
TLC plate : Silica gel $60F_{254}$ (E. Merck)

Table 2. Elemental analysis, molecular formula and acyl side chains.

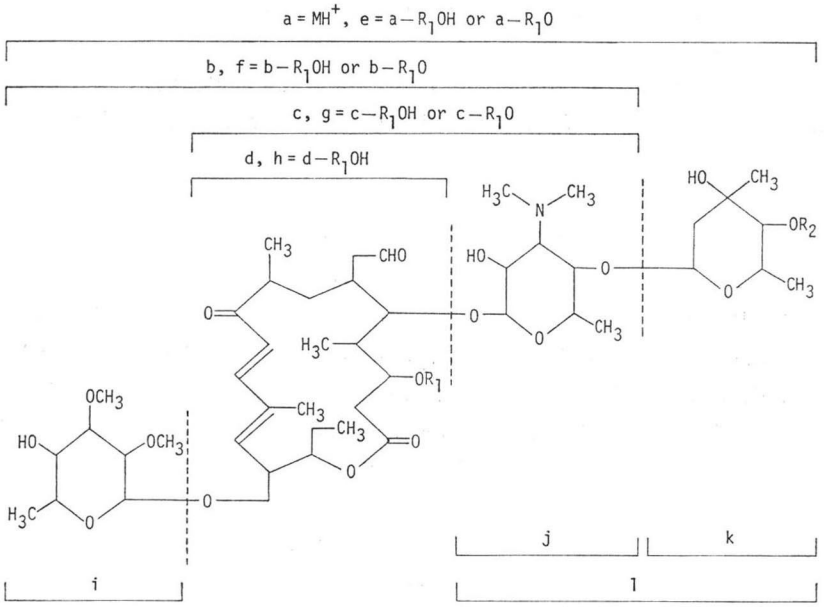
Compound	Mol. Wt.	Formula	Elemental analysis (%) Found (Calculated)				Organic acid
			C	H	N	O	
II	957	$C_{45}H_{79}NO_{18}$	60.25 (60.17)	8.27 (8.31)	1.50 (1.56)	29.98 (30.06)	Acetic acid
III	971	$C_{49}H_{81}NO_{18}$	60.45 (60.54)	8.46 (8.40)	1.49 (1.44)	29.59 (29.62)	Propionic acid
IV	985	$C_{50}H_{83}NO_{18}$	61.03 (60.90)	8.55 (8.48)	1.48 (1.42)	28.94 (29.20)	*n*-Butyric acid
V	999	$C_{51}H_{85}NO_{18}$	61.33 (61.24)	8.65 (8.57)	1.46 (1.46)	28.56 (28.79)	Isovaleric acid
VI	1,027	$C_{52}H_{85}NO_{19}$	60.70 (60.75)	8.39 (8.33)	1.40 (1.36)	29.51 (29.56)	Acetic acid, *n*-butyric acid
VII	1,041	$C_{53}H_{87}NO_{19}$	61.05 (61.08)	8.33 (8.41)	1.39 (1.34)	29.23 (29.17)	Acetic acid, isovaleric acid
VIII	1,041	$C_{53}H_{87}NO_{19}$	61.05 (61.08)	8.37 (8.41)	1.33 (1.34)	29.25 (29.17)	Propionic acid, *n*-butyric acid
IX	1,055	$C_{54}H_{89}NO_{19}$	61.54 (61.40)	8.46 (8.49)	1.31 (1.33)	28.70 (28.78)	Propionic acid, isovaleric acid

II, **VI** and **VII** is also shown by the peak at around 1240 cm^{-1}.

^{1}H-NMR data of all acylated derivatives contain the signals of aldehyde, olefinic, methoxy, N-methyl and 12-methyl groups which are characteristic of tylosin (**I**). In addition, **II**, **VI** and **VII** reveal the O-acetyl peak at δ 2.1 (3H, s). The aldehyde proton is seen at δ 9.7 in **IV** and **V**, and at around δ 9.6 in **II**, **III**, **VI**, **VII**, **VIII** and **IX**. A similar shift was reported in spiramycin and leucomycin.[5] The signals of isovaleryl group were seen at δ 0.97 (6H, d, J=6 Hz) and δ 2.28 (2H, d, *ca.* J=4 Hz) in **V**, **VII** (Fig. 5) and **IX**.

Comparison of the ^{13}C-NMR of **II** and **VII** with that of **I**, shows that **II** contains two (170.4 and 21.2 ppm) and **VII** seven (170.4, 21.2, 173.2, 43.4, 25.6, 22.5 and 22.5) carbon atoms more than **I**. These carbon atoms can be assigned to an acetyl group in **II**, and an acetyl and an isovaleryl group in **VII**. The effect of 3-acetylation is shown in the marked upfield shift of the signal attributable to C-1 (174.2 ppm) of **I**. This is shifted to 170.8 ppm in **II** and 170.9 ppm in **VII**. A similar shift has been reported in leuco-

Fig. 3. CI-mass fragmentation patterns of tylosin acyl derivatives.



mycins by ŌMURA *et al.*[6] The 4''-isovalerylation causes the downfield shift of 4''-carbon (75.3 for **I** *versus* 77.2 for **VII**) with a simultaneous upfield shift of 5''-carbon (66.2 for **I** *versus* 63.7 for **VII**) and 6''-carbon (18.3 for **I** *versus* 17.8 for **VII**).

The results of CI-mass spectral analysis are summarized in Table 3 and Fig. 3. **II**, **III** and **VII** gave MH^+ as a weak peak, and **IV**, **V**, **VIII** and **IX** did not give MH^+ peaks. But **IV**, **V**, **VI**, **VIII** and

Table 3. CI-Mass spectral data for tylosin (**I**) and derivatives (**II**~**IX**).

Compound	a (MH^+)	b	c	d	e	f	g	h	i	j	k	l	Base peak
I	916 (2)	772 (30)	598 (4)	407 (13)	—	—	—	—	175 (37)	192 (43)	145 (33)	336 (19)	101 (100)
II	958 (0.2)	815 (1)			898 (10)	755 (30)	581 (6)	389 (5)	175 (13)	192 (28)	145 (15)	336 (3)	127 (100)
III	972 (0.3)	828 (3)	654 (1)		898 (12)	754 (77)	580 (20)	389 (11)	175 (35)	192 (28)	145 (30)	336 (6)	174 (100)
IV	968* (0.3)	772 (3)		407 (36)	—	—	—	—	175 (92)	192 (53)	215 (100)	406 (74)	215 (100)
V	982* (0.6)	754* (3)		407 (15)	—	—	—	—	175 (86)	192 (29)	229 (100)	420 (38)	229 (100)
VI	1,010* (0.5)	796* (2)			968 (2)	754 (9)	580 (4)	389 (12)	175 (80)	191 (23)	215 (60)	406 (15)	127 (100)
VII	1,042 (0.4)	814 (2)			982 (9)	754 (34)	580 (7)	389 (14)	175 (26)	192 (11)	229 (38)	420 (9)	109 (100)
VIII	1,024* (0.3)	810* (0.1)			967 (0.1)	754 (9)	580 (5)	389 (13)	175 (72)	191 (22)	215 (50)	406 (13)	109 (100)
IX	1,038*	810* (4)			981 (6)	754 (52)	580 (6)	389 (24)	175 (65)	191 (20)	229 (67)	420 (30)	109 (100)

CI-Mass spectra were recorded using methane or isobutane as reactant gas. Number in parenthesis shows the relative intensity.

* shows the dehydrated ion peak.

IX gave their dehydrated peaks. In addition, the 3-deacylation occurred and the abundant peaks of deacylated fragment were observed. The 4''-acyl group was shown to be stable, and acyl mycarose and acyl disaccharide peaks were detected.

FD-mass spectrometry, in contrast, gave very intense and almost sole MH^+ peak for each acyl derivatives. Thus, molecular weights of the eight acylated tylosins were determined from FD-mass spectral data.

Structural and Chemical Studies

Structure of II

Based on the ^{13}C-NMR and the release of one mole of acetic acid on alkaline hydrolysis, **II** was shown to be a monoacetylated tylosin. In order to determine the location of the acetyl group, **II** was subjected to a mild acid hydrolysis which gave mycarose and a monoacetyldesmycosin (**XIV**). In the ^{1}H-NMR spectrum of **XIV** (Fig. 4), a doublet at δ 5.1 (1H, J=10 Hz) and a singlet at δ 2.1 (3H) which are not observed in the spectrum of desmycosin (**XIII**)[7] were observed. The signal at δ 5.1 can be assigned to the C-3 methine proton which shifts to a lower magnetic field because of the acetylation of the C-3 hydroxyl group, as is the case in tetraacetyldesmycosin.[8] This was supported by the spin decoupling experiment: by the irradiation at δ 2.5 (C-2H$_A$), a doublet type signal at δ 5.1 collapsed to a broad singlet. Moreover, in the EI-mass spectrum of **XIV** which showed the molecular ion peak at m/z 813, the acetylated aglycone peak was observed at m/z 449, indicating the acetylation of the aglycone part of desmycosin, that is, the 3-O-acetylation. Thus, **II** which was shown to be a monoacetyl derivative of tylosin by molecular weight determination was determined to be 3-acetyltylosin.

Structure of V

Release of one mole of isovaleric acid on alkaline hydrolysis and its molecular weight indicated that **V** should be a monoisovaleryltylosin. After hydrolysis under a mild acid condition, **XIII** and an acylated

Fig. 4. ^{1}H-NMR spectrum of 3-acetyldesmycosin (**XIV**) in $CDCl_3$.

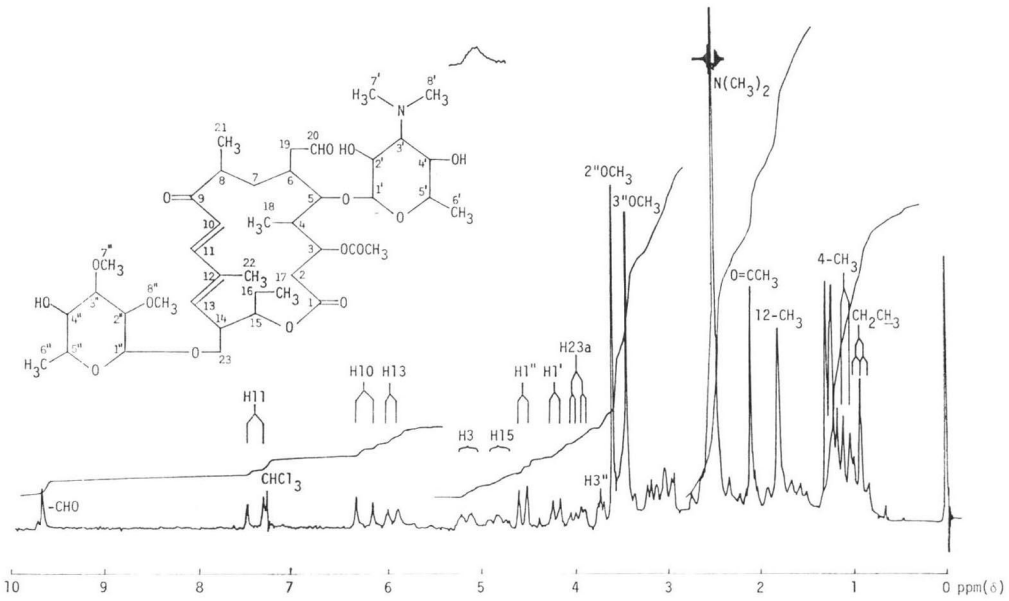
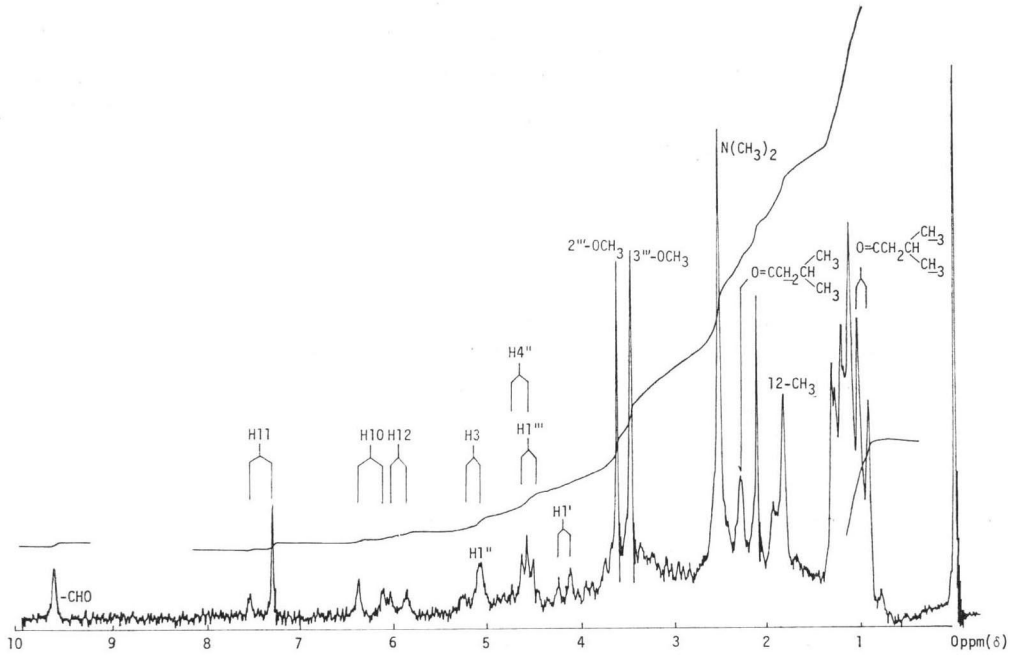


Fig. 5. ^{1}H-NMR spectrum of 3-acetyl-4″-isovaleryltylosin (**VII**) in $CDCl_3$.



mycarose were recovered. After methylation of the latter, the mycarose moiety was identified to be a methyl-4-O-isovalerylmycarose (**XV**) by comparison with an authentic sample which was prepared from leucomycin A_3.[9] Thus, **V** was concluded to be 4″-isovaleryltylosin.

Structure of VII

On alkaline hydrolysis of **VII**, the release of each one mole of acetic acid and isovaleric acid was determined by gas chromatography. As 3-acetyldesmycosin (**XIV**) and 4-O-isovalerylmycarose were recovered on mild acid hydrolysis, **VII** was shown to be 3-acetyl-4″-isovaleryltylosin. This was also supported by the detection of the protonated molecular ion peak at *m/z* 1,042 and the fragmentation pattern in the CI-mass spectrum (Fig. 3 and Table 3) and the analysis of ^{13}C-NMR spectrum. In the ^{1}H-NMR spectrum of **VII** (Fig. 5), the irradiation at δ 2.5 converted the peak at δ 5.1 to a broad singlet, indicating that the 3-hydroxyl group was acylated. The signals observed at δ 0.97 and 2.28 were attributed to the dimethyl and methylene protons of the isovaleryl group respectively.

Structure of III, IV, VI, VIII and IX

Structures of the remaining derivatives were determined by comparison of their behavior in gas chromatography, ^{1}H-NMR spectrometry and CI-mass fragmentation (Tables 2 and 3, and Fig. 3) with those of **II**, **V** and **VII**. In the CI-mass spectra, as was reported for spiramycin and niddamycin,[10] the most abundant fragment ions resulted from the cleavage of glycosidic bonds together with dehydration. The C-3 acyl groups of these tylosin derivatives are easily lost before the ion peaks of the sugars were detected, while C-4″ acyl groups are stable enough to give the fragment ions corresponding to acyl mycarose and protonated acyl mycarosylmycaminose. Thus, **III**, **IV**, **VI**, **VIII** and **IX** were determined to be 3-propionyltylosin, 4″-butyryltylosin, 3-acetyl-4″-butyryltylosin, 3-propionyl-4″-butyryltylosin and 3-propionyl-4″-isovaleryltylosin respectively.

Tylosin is a basic 16-membered macrolide compound having five hydroxyl groups among which the four can be chemically acylated under usual conditions.[10,11] These chemically acylated derivatives of tylosin which have been described in the patent specifications[12] are different from acyl derivatives reported in this present paper in their physico-chemical properties. The results of the structural studies presented in this paper clearly indicate that *S. thermotolerans* acylates tylosin at the 3- and 4″-positions selectively, but not at the 2′- and 4‴-positions.

Experimental

General methods

Melting points (uncorrected) were measured on a Kofler hot stage apparatus. Specific rotations were determined in methanol solutions by a JASCO-181 automatic polarimeter. UV and IR absorption spectra were recorded in a Hitachi EPS-3T spectrometer and in a Hitachi EPI-G2 (KBr tablets) respectively. TLC was performed on a silicagel plate (SILICAGEL $60F_{254}$; E. Merck, Darmstadt) and macrolide spots were revealed by sulfuric acid reaction.

NMR and mass spectrometry

^{1}H-NMR and ^{13}C-NMR spectra were measured in $CDCl_3$ by a Hitachi R-24 spectrometer at 60 MHz, a Varian EM-390 spectrometer at 90 MHz and a Varian XL-100 spectrometer at 25.2 MHz respectively. Chemical shifts were given in ppm values from tetramethylsilane as the internal standard.

EI-mass spectra and CI-mass spectra were recorded by a Hitachi RMU-6M mass spectrometer. EI-mass spectrometry was carried out at 70 eV by direct inlet system at approximately 180°C. The temperature of the ion source was 190°C. Methane or isobutane was used as the reactant gas for CI-mass spectrometry. FD-mass spectra were taken by a Hitachi M-80 mass spectrometer.

Preparation of acylated tylosin derivatives

For a preparation of acylated tylosins, a high acylase strain numbered as 8254 was selected by single spore isolation from the type culture of *S. thermotolerans* ATCC 11416 after treatment with N-methyl-N′-nitro-N-nitrosoguanidine. *S. thermotolerans* 8254 was cultured in a jar fermentor containing 15 liters of medium (glucose, 20 g/liter; soybean meal, 5 g/liter; yeast extract, 5 g/liter; peptone, 5 g/liter; K_2HPO_4, 0.5 g/liter; $MgSO_4 \cdot 7H_2O$, 0.5 g/liter; pH 7.0 prior to autoclaving) at 36°C for 24 hours under aerobic conditions. At this time, tylosin was added at a concentration of 1,000 μg/ml and the fermentation was continued for 6 hours. Then, 3-acetyltylosin (**II**) was preferentially formed together with a small amount of 3-acetyl-4″-isovaleryltylosin (**VII**). The addition of L-leucine which is a common precursor for the isovaleryl group was proved to increase 4″-isovaleryltylosins. When DL-α-aminobutyric acid and DL-norvaline were supplemented to the culture broths, 3-propionyltylosins and 4″-butyryltylosins were formed respectively, but their yields were far less than those of 3-acetyl- and 4″-isovaleryltylosins. In order to suppress the influence of endogenously available pool of the acetyl and isovaleryl donors as much as possible, washed mycelia of *S. thermotolerans* 8254 were employed for the production of 3-propionyltylosin (**III**), 4″-butyryltylosin (**IV**) and 4″-isovaleryltylosin (**V**). For the preparation of 3-acetyl-4″-butyryltylosin (**VI**), **II** was added instead of tylosin. For the preparation of 3-propionyl-4″-butyryltylosin (**VIII**) and 3-propionyl-4″-isovaleryltylosin (**IX**), **III** was added.

Transformation products were isolated by an established procedure for basic macrolides. The reaction mixture was filtered at pH 4.0 through a filter press to give *ca.* 15 liters of filtrate. The filtrate was adjusted to pH 8.0 and extracted at 30°C with 5 liters of ethyl acetate. The extract was cooled to 5°C and back-extracted with 2.5 liters of 0.1 M citrate buffer (pH 3.5). The aqueous extract was adjusted to pH 8.0 at 30°C and extracted again with 1.25 liters of ethyl acetate. Evaporation of the ethyl acetate extract gave a pale yellow powder. This powder was purified by silica gel column chromatography, with eluent containing stepwise increases in the concentration of methanol in benzene (2: 98 ~ 10: 90). Eluate fractions containing the desired product were evaporated to dryness, the residue was dissolved in a mixture of ethyl ether and isopropyl ether (5: 2) and allowed to crystallize at 5°C.

Alkaline hydrolysis of **II**~**IX**

The established GLC method for the analysis of 16-membered macrolides was employed[2]: 20 mg of a sample to be analyzed was hydrolyzed in 2 ml of 0.5 N KOH in ethanol for 20 minutes at 70~75°C and evaporated to dryness *in vacuo*. The residue was dissolved in 1 ml of 3 N HCl and extracted with 1 ml of ethyl ether. Two microliters of the ethyl ether layer were injected onto a glass column (3.0 mmϕ ×1.5 m) containing 10% PEGA and 1% H_3PO_4 on Chromosorb W in a Yanagimoto G-1800 gas-liquid chromatograph (injection port 250°C, column 120°C). The retention times of relevant fatty acids observed under the above conditions were as follows: acetic acid 1.73 minutes; propionic acid 2.58 minutes; *n*-butyric acid 3.80 minutes; and isovaleric acid 4.50 minutes.

Partial acid hydrolysis of **II**

One gram of **II** was suspended in 25 ml of distilled water and the suspension was adjusted to pH 2.7 with 10% HCl. After standing for 45 hours at room temperature, the reaction mixture containing **XIV** and mycarose was adjusted to pH 8.5 with dilute NaOH, and **XIV** was extracted twice with 10 ml each of $CHCl_3$. The extract layers ($CHCl_3$) were combined and evaporated to dryness *in vacuo*. The evaporation residue was dissolved in a small amount of benzene and chromatographed on a silica gel column (180ϕ×400 mm, Wako silica gel C-200) using a solvent mixture (*n*-hexane - acetone - methanol - benzene - ethyl acetate, 30: 10: 8: 25: 20). The eluate fractions (10 ml fraction) containing **XIV** were combined and evaporated to dryness *in vacuo* to yield 300 mg of colorless powder of 3-acetyldesmycosin (**XIV**).

XIV: m.p., 120.5~122.5°C; $[\alpha]_D^{20}$ −11.5° (*c* 2.0, MeOH); λ_{max}^{MeOH} 282 nm, $E_{1cm}^{1\%}$=265; Anal. Calcd. for $C_{41}H_{67}NO_{15}$ (MW 813): C 59.90, H 8.42, N 1.75, Found: C 59.96, H 8.38, N 1.73.

From the raffinate layer (aqueous layer), mycarose was isolated. The identity of this compound was proved by comparison with authentic mycarose obtained from tylosin.

Partial acid hydrolysis of **V**

Two grams of **V** was dissolved in 30 ml of 0.35 N HCl and allowed to stand at 7°C for 24 hours. After adjustment to pH 4.0 with 5 N NaOH, the solution was subjected to repeated extraction with 50 ml each of $CHCl_3$. The $CHCl_3$ extracts were combined, washed with saturated NaCl solution, dried over Na_2SO_4 and evaporated to dryness *in vacuo*. The residue was dissolved in 30 ml of 1% HCl in MeOH and refluxed for 2 hours. After the solution was cooled and adjusted to pH 4.0 with 5 N NaOH, methanol was removed by evaporation *in vacuo*. The residue was dissolved in 50 ml of ethyl ether, washed with 20 ml each of water and saturated NaCl solution and dried over Na_2SO_4 before evaporation. The evaporation under reduced pressure gave a mixture of α- and β-methyl-4-O-isovalerylmycaroside (**XV**) as colorless liquid (0.1 g).

XV: b.p. 115°C/3 mmHg; Rf 0.88 and 0.83 in a solvent system (*n*-hexane - ethyl acetate - benzene - acetone - methanol, 30: 20: 25: 10: 8). Anal. Calcd. for $C_{13}H_{24}O_5$ (MW 260): C 59.98, H 9.18, Found: C 60.02, H 9.26. IR (KBr) 3500, 2955, 1738, 1370, 1170 and 1050 cm^{-1}.

Desmycosin (**XIII**) was isolated from the raffinate layer (hydrolysate after chloroform extraction) and identified by its physico-chemical properties.[4]

Partial acid hydrolysis of **VII**

VII (2.0 g) was treated and the same manner as for **V** to provide 0.6 g of 3-acetyldesmycosin (**XIV**) and 0.3 g of methyl-4-O-isovalerylmycaroside (**XV**).

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