NEW TYLOSIN ANALOGS PRODUCED BY MUTANTS OF *STREPTOMYCES FRADIAE*

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2'''-Demethoxytylosin (component **IIIc**), 2'''-demethoxy-4'''-*epi*-tylosin (component **IIId**) and 2'''-*O*-demethyltylosin (component **Vb**) were produced by blocked mutant strains of *Streptomyces fradiae*. Fermentation, isolation, structure determination and biosynthetic considerations of these tylosin analogs are described.

Tylosin is a 16-membered macrolide antibiotic produced by *Streptomyces fradiae*[1] and consists of tylonolide, mycaminose, mycarose and mycinose. Recent mutagenic studies[2~4] revealed many biosynthetic precursors from protylonolide to tylosin, resulting in the elucidation of the route of tylosin biosynthesis[5,6]. These precursor compounds serve not only to demonstrate the biosynthetic pathway of tylosin, but also to collect information useful for improvement of fermentation yields and for elucidation of the structure-activity relationship among macrolides, which will lead to future development of clinically effective macrolide derivatives.

In continuing mutagenic studies of *Streptomyces fradiae*, a tylosin producer, the structures of four tylosin precursors (components **II**, **IIIa**, **IIIb** and **IVa**) were elucidated[4], but components **IIIc** and **IIId** were not yet structurally determined. In addition, a new mutant strain (TK-22563) was found to produce a novel analog (component **Vb**). This paper describes the fermentation, isolation, structure determination and antimicrobial activity of these three tylosin analogs and discusses their possible roles in tylosin biosynthesis.

Materials and Methods

Media and Cultivation Conditions

Sporulation medium contained malt extract 10 g, yeast extract 4 g, glucose 4 g and agar 20 g in 1 liter deionized water, and was adjusted to pH 7.0. Vegetative medium consisted of soybean meal 20 g, soluble starch 20 g, yeast extract 3 g, calcium carbonate 3 g, magnesium sulfate ($7H_2O$) 1 g and dipotassium phosphate 1 g in 1 liter deionized water, and was adjusted to pH 7.0. For tylosin analog fermentation, medium composed of rape oil 60 g, dried yeast 30 g, yeast extract 10 g, dipotassium phosphate 1 g, calcium carbonate 1 g and magnesium sulfate ($7H_2O$) 1 g in 1 liter deionized water (pH 7.5) was employed.

N-Methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG)-treated spores were grown on sporulation medium at 30°C for 8 days and each colony was transferred on a slant of sporulation medium and sporulated

at 30°C for 8~14 days. One loopful of mature spores was inoculated into a 250-ml Erlenmeyer flask containing 50 ml of vegetative medium and cultured at 30°C for 2 days on a rotary shaker (7 cm stroke; 220 rpm). The seed culture (2 ml) was transferred into 35 ml fermentation medium in a 250-ml Erlenmeyer flask and fermented at 30°C for 8 days on a rotary shaker (7 cm stroke; 270 rpm). The broth filtrate was subjected to silica gel TLC analysis, as explained below.

For large-scale production, a 20-liter jar fermentor containing 10 liters of fermentation medium was inoculated with the seed culture equal to 5% of its volume, and aerobically fermented at 30°C and 400 rpm (aeration rate: 0.5 vvm).

NTG Mutagenesis

Mature spores of *Streptomyces fradiae* NRRL 2702 or derivatives from a 2-week-old agar culture were suspended in 10 ml of sterile water and dispersed well by ultrasonication under ice-cooling. After passing through a 3G-3 glass filter, 1 ml of the filtrate was diluted with 9 ml of 10 mM phosphate buffer, pH 8.0, containing 800 μg/ml of NTG. The spore suspension was incubated at 30°C for 60 minutes and then centrifuged. The NTG-treated spores were resuspended in 10 ml of sterile water, plated on sporulation medium, and incubated at 30°C for 8 days.

New mutant numbered TK-22563 produced components **Va** and **Vb** without any other components (TLC analysis).

Silica Gel TLC Analysis of Tylosin and Analogs

Macrolides in 1 ml of broth filtrate were extracted at pH 9.0 with 1 ml of ethyl acetate. An aliquot of this extract (10 μl) was spotted onto a silica gel plate (pre-coated Silica gel 60 F_{254} plate, E. Merck, Darmstadt) and developed in solvent system A (benzene - acetone (1:2)) or B (chloroform - methanol - 25% ammonia (15:1:0.1)). The TLC plate was dipped in 10% sulfuric acid and heated at 105°C for 15 minutes. Rf values in solvent systems A and B were as follows: Tylosin, 0.26 and 0.43; component **IIIa**[4], 0.23 and 0.23; component **IIIb**[4], 0.31 and 0.62; component **IIIc**, 0.26 and 0.47; component **IIId**, 0.24 and 0.38; component **Va**, 0.03 and 0.04; component **Vb**, 0.17 and 0.21, respectively.

Isolation and Purification of Macrolide Compounds

The fermentation broth (10 liters) of mutant YO-9010 was mixed with 2% Hyflo Super Cel and the filtrate was extracted at pH 9.0 twice, each time with a half volume of ethyl acetate. Macrolide compounds in the organic extract were re-transferred twice into ice-cold water at pH 4.1 (2.5 liters each time). For quick rinsing, the macrolide compounds in the aqueous extract were again extracted at pH 9.0 twice, each time with 2.5 liters of ethyl acetate, and the ethyl acetate extract was concentrated to *ca.* 20 ml in a rotary evaporator. Dropwise addition of the concentrate into 400 ml of *n*-hexane gave a crude mixture of components **IIIa**, **IIIb**, **IIIc** and **IIId**. As described previously[4], components **IIIa** and **IIIb** were removed by silica gel column chromatography, and components **IIIc** and **IIId** were separated by preparative silica gel TLC (0.5 mm in thickness) with a solvent system of chloroform - methanol - 25% ammonia (15:1:0.1). One batch of the jar fermentation broth yielded 4.5 g of component **IIIa**, 0.18 g of component **IIIb**, 0.12 g of component **IIIc** and 0.02 g of component **IIId**.

Components **Va** (2‴-*O*-demethylmacrocin) and **Vb** of mutant strain TK-22563 were similarly isolated. The eluting solvent for silica gel column (300 ml) chromatography was toluene - acetone (3:2) for component **Vb** and toluene - acetone (1:1) for component **Va**. The yields of components **Va** and **Vb** from a 10-liter batch were 3.7 g and 0.27 g, respectively.

Preparation of Methyl β-D-Cymaroside (1) and Methyl β-D-Sarmentoside (3)

Component **IIId** (0.4 g) was dissolved in 25 ml of 0.05 N HCl and the pH was adjusted to 2.0 with 1 N HCl. The acid solution was allowed to stand at room temp for 20 hours. After adjustment of the pH to 9.0 with 0.2 N NaOH, the solution was extracted twice with chloroform (30 ml each time), leaving 60 mg of L-mycarose in the aqueous solution. The chloroform extract was dried over sodium sulfate and filtered. The organic filtrate was evaporated to dryness under reduced pressure to yield 310 mg of demycarosyl component **IIId**. This evaporated residue was dissolved in 25 ml of 1% HCl - methanol and allowed to stand overnight at room temp. After adjustment of the pH to 4.0 with 0.2 N NaOH followed by dilution with 20 ml of water, the methanol was removed by evaporation. The aqueous solu-

tion was extracted twice with chloroform (30 ml each time), and the combined chloroform extracts were dried over sodium sulfate and then evaporated to dryness. The evaporation residue was subjected to silica gel column (15 ml) chromatography with an eluent system of chloroform - methanol (300:1), yielding 40 mg of methyl β-D-sarmentoside (**3**).

Methyl β-D-cymaroside (**1**; 75 mg) was similarly prepared from 900 mg of component **IIIc**.

Acetylation of Methyl β-D-Cymaroside (1) and Methyl β-D-Sarmentoside (3)

Methyl β-D-cymaroside (**1**; 40 mg) in 1.5 ml pyridine was mixed with 1.5 ml acetic anhydride and stirred at 8°C for 20 hours. Ice-water (10 ml) was added to the reaction mixture to decompose excess acetic anhydride, and the acetylated sugar was extracted twice with benzene (20 ml each time). The benzene extracts were combined and rinsed with satd sodium bicarbonate and then with satd sodium chloride, dried with sodium sulfate, and evaporated to dryness. Purification by silica gel column chromatography (15 ml silica gel; solvent system: benzene - acetone, 100:1) gave 15 mg of methyl 4-*O*-acetyl-β-D-cymaroside (**2**).

Methyl 4-*O*-acetyl-β-D-sarmentoside (**4**; 8 mg) was obtained from 70 mg of **3**, as described above.

Bioconversion of Components Va and Vb

S. fradiae mutants YT-A, YT-B, YT-I, YT-L and YT-Q, which produced no macrolide compounds, were preliminarily tested using component **Va** as substrate. Mutants YT-I and YT-L were selected for their ability to methylate 2‴-*O*-demethylmacrocin (=component **Va**) to tylosin, while mutants YT-A, YT-B and YT-Q could not presumably because of some defect in methylation.

2‴-*O*-Demethyltylosin (component **Vb**) was added to the 24-hour-old cultures of mutant YT-I, YT-L or TK-22563 at 200 μg/ml and fermented for a further 48 hours. After mycelia were removed, the filtrates were extracted at pH 9 with ethyl acetate and analyzed by silica gel TLC.

General Methodology

Melting points were determined on a Yanagimoto micro melting point apparatus and are uncorrected. UV absorption spectra were measured with a Hitachi 200-20 spectrophotometer; optical rotations, with a Jasco DIP-181 digital polarimeter; field desorption mass spectra (FD-MS), with a Hitachi RMU-7M mass spectrometer; ^{1}H NMR spectra, with a Varian XL-100 spectrometer at 100 MHz or with a Varian EM-390 spectrometer at 90 MHz; and ^{13}C NMR spectra, with a Varian XL-100 spectrometer at 25.2 MHz. Silica gel for column chromatography was Wakogel C-200 (Wako Pure Chemical Industries, Ltd.).

Results and Discussion

Structure Determination

Physico-chemical properties of components **IIIc**, **IIId** and **Vb** are summarized in Table 1.

UV spectrometry demonstrated that the three components had the dienone chromophore at 283 ~ 284 nm. The molecular weights of components **IIIc**, **IIId** and **Vb** were determined to be 886, 886 and 902 by FD-MS.

^{1}H NMR spectrometry revealed the signals of aldehyde, olefin, *N*-dimethyl and C-12 methyl in components **IIIc**, **IIId** and **Vb**, which are all present in the spectrum of tylosin. It is important to note, however, that the three new analogs had only one *O*-methyl signal, while tylosin gives two. In addition, components **IIIc** and **IIId** showed the double doublet signal of 1‴-H, indicating that they had a proton at C-2‴ instead of *O*-methyl.

^{13}C NMR spectrometric data in Table 2 show that, except for the third sugar moiety (mycinose), there are no substantial differences among the new analogs and tylosin. Proton decoupling revealed that the three components were composed of 45 carbon atoms (one carbon atom less than tylosin). Components **IIIc** and **IIId** were clearly differentiated from tylosin in the third sugar moiety. One 2‴-

Table 1. Physico-chemical properties of components **IIIc**, **IIId**, **Vb** and tylosin.

	IIIc	**IIId**	**Vb**	Tylosin
MP (°C)	122~126	133~137	124~128	128~132
$[\alpha]_D^{23}$ (MeOH)	−36.6° (*c* 0.77)	−45.9° (*c* 0.67)	−38.9° (*c* 0.50)	−46° (*c* 1.00)
UV λ_{max}^{MeOH} nm ($E_{1cm}^{1\%}$)	284 (228)	284 (223)	283 (245)	282 (245)
FD-MS (*m/z*, $(M+H)^+$)	886	886	902	916
Formula	$C_{45}H_{75}NO_{16}$	$C_{45}H_{75}NO_{16}$	$C_{45}H_{75}NO_{17}$	$C_{46}H_{77}NO_{17}$
Elemental *Anal*				
Found (calcd) C	61.22 (60.99),	61.13 (60.99),	59.84 (59.91),	59.68 (60.31),
H	8.60 (8.53),	8.75 (8.53),	8.40 (8.38),	8.53 (8.47),
N	1.55 (1.58).	1.49 (1.58).	1.54 (1.55).	1.55 (1.53).
Rf (silica gel TLC)*				
Solvent system A	0.26	0.24	0.17	0.26
Solvent system B	0.47	0.38	0.21	0.43
^1H NMR (ppm)†				
3‴-*O*-CH_3	3.43 s	3.39 s	3.65 s	3.62 s
1‴-H ($J_{1''',2'''ax}$)	4.60 dd (9)	4.60 dd (9)	4.52 d (7.5)	4.56 d (7.5)
2‴-H_{ax} ($J_{2''',3'''}$)	—	—	3.48 dd (3)	3.04 dd (3)
2‴-H_{eq} ($J_{1''',2'''}$)	— (2)	— (3)		
3‴-H ($J_{3''',4'''}$)	—	—	3.76 dd (3)	3.76 dd (3)

* Pre-coated Silica gel 60 F_{254} plates, E. Merck, Darmstadt; solvent system A: benzene - acetone (1:2), solvent system B: chloroform - methanol - 25% ammonia (15:1:0.1).

† In $CDCl_3$; coupling constants in Hz in parentheses.

—: Unassignable.

Table 2. ^{13}C NMR data of components **IIIc**, **IIId**, **Vb** and tylosin.

Carbon	**IIIc**	**IIId**	**Vb**	Tylosin	Carbon	**IIIc**	**IIId**	**Vb**	Tylosin
1	174.0	174.0	174.1	174.2	1′	103.8	103.8	104.0	104.0
2	39.4	39.4	39.5	39.5	2′	71.8[a]	71.8[a]	71.8[a]	71.9[a]
3	68.2[a]	68.8[a]	68.8[a]	68.0[a]	3′	68.9[a]	69.2[a]	68.9[a]	69.0[a]
4	45.1[b]	45.1[b]	45.2[b]	45.2[b]	4′	76.5	76.5	76.5	76.6
5	81.5	81.5	81.6	81.5	5′	73.2	73.2	73.3	73.3
6	32.2	32.1	32.3	32.1	6′	19.1	19.1	19.1	19.1
7	32.9	32.8	33.0	32.9	$N(CH_3)_2$	42.0	42.0	42.0	42.0
8	40.2[b]	40.1[b]	40.2[b]	40.4[b]	1″	96.5	96.5	96.6	96.7
9	202.7	202.8	202.7	203.2	2″	41.0	41.0	41.0	41.0
10	118.9	118.8	119.3	118.9	3″	69.5	69.5	69.5	69.6
11	148.1	148.1	147.8	148.4	4″	75.3	75.3	75.2	75.3
12	135.1	135.1	135.4	135.1	5″	66.1	66.1	66.1	66.2
13	142.4	142.2	141.5	142.5	6″	18.3	18.3	18.3	18.3
14	44.7[b]	44.6[b]	44.4[b]	44.8[b]	7″	25.4	25.4	25.4	25.5
15	75.0	75.0	75.1	75.3	1‴	98.2	99.3	101.4	101.3
16	25.7	25.6	25.8	25.5	2‴	33.4	30.5	72.8	82.1
17	9.1	9.0	9.0	9.1	3‴	77.2	78.2	80.2	80.0
18	9.7	9.7	9.7	9.7	4‴	72.5	68.0	72.9	72.9
19	43.8	43.8	43.9	43.9	5‴	71.0	69.3	71.2	70.7
20	203.2	203.2	203.1	203.4	6‴	18.3	16.4	17.8	17.8
21	17.4	17.4	17.2	17.4	2‴-OCH_3				59.8
22	13.1	13.1	13.0	13.0	3‴-OCH_3	57.2	57.0	61.9	61.8
23	68.9	69.3	69.4	69.2					

^{13}C NMR spectra were recorded at 25.2 MHz in $CDCl_3$. Chemical shifts are given in ppm relative to $(CH_3)_4Si$ as internal standard.

a, b Assignments may be interchanged.

Table 3. ^{1}H NMR spectral data of sugar derivatives **1**~**4** at 90 MHz.

Sugar derivative*	Chemical shift (ppm)								
	1-H ($J_{1,2ax}$)	2-H$_{ax}$ ($J_{2ax,2eq}$)	2-H$_{eq}$ ($J_{1,2eq}$)	3-H ($J_{2ax,3}$)	4-H ($J_{3,4}$)	5-H ($J_{4,5}$)	6-H ($J_{5,6}$)	OCH_3	$OCOCH_3$
Methyl *β*-D-cymaroside (**1**)	4.56	1.55	2.25	3.63	—	—	1.30	3.43,	
	(9.3)	(14.3)	(2.7)	(3)	(3.3)		(6.2)	3.47	
Methyl 4-*O*-acetyl-	4.66	1.63	2.16	3.76	4.56	3.98	1.22	3.35,	2.05
β-D-cymaroside (**2**)	(8.6)	(13.6)	(2.6)	(2.8)	(3)	(9)	(6.3)	3.44	
Methyl	4.54	1.70	1.90	—	—	3.93	1.26	3.37,	
β-D-sarmentoside (**3**)	(9.2)	(14.5)	(3.4)	(3.2)		(1.4)	(6.8)	3.47	
Methyl 4-*O*-acetyl-	4.60	1.70	1.93	—	4.73	4.00	1.20	3.42,	2.11
β-D-sarmentoside (**4**)	(9.5)	(14)	(2.7)	(3.2)	(3.2)	(1.3)	(6.6)	3.49	

* In $CDCl_3$; coupling constants in Hz in parentheses.
—: Unassignable.

Fig. 1. Structures of methyl glycoside derivatives prepared from the third sugar moiety of components **IIIc** and **IIId**.

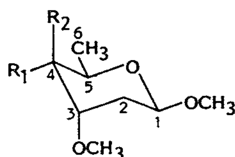
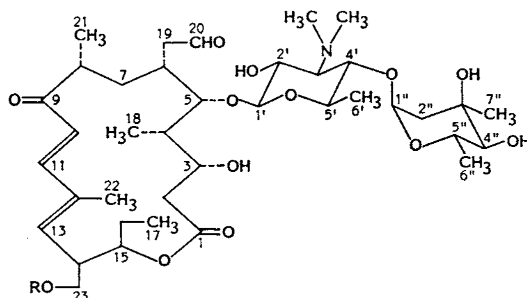
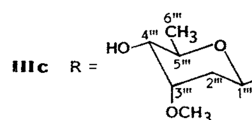
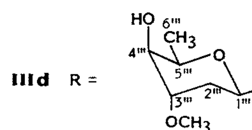
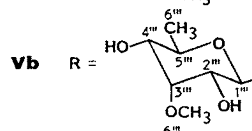


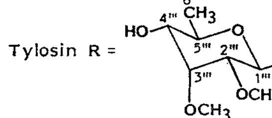
Fig. 2. Structures of components **IIIc**, **IIId**, **Vb** and tylosin.











CH-O carbon of tylosin at 82.1 ppm disappeared and instead one CH_2 carbon appeared at 33.4 ppm in component **IIIc** and at 30.5 ppm in component **IIId**. Accordingly, one anomeric, one methylene, three methine, one methyl and one *O*-methyl carbons were allocated to the third sugar moiety of components **IIIc** and **IIId**.

The planar structure of components **IIIc** and **IIId** was thus considered to be 2‴-demethoxytylosin. For determination of the configuration of the third sugar moiety in components **IIIc** and **IIId**, methyl monoacetyl glycosides (**2** and **4**) corresponding to the third sugar moieties were prepared from demycarosyl components **IIIc** and **IIId** *via* **1** and **3**, respectively. From the ^{1}H NMR spectrometric data in Table 3, methyl glycosides **1** and **3** were concluded to be methyl 2,6-dideoxy-3-*O*-methyl-*β*-D-ribohexopyranoside (=methyl *β*-D-cymaroside)[7] and methyl 2,6-dideoxy-3-*O*-methyl-*β*-D-xylohexopyranoside (=methyl *β*-D-sarmentoside), respectively. This conclusion was also supported by hydrolysis of methyl glycosides **1** and **3** which yielded D-cymarose ($[\alpha]_D^{23}$ in water: +52° (found);

+54° (reported)) and D-sarmentose (+17° (found); +15.8° (reported))[8], respectively. Thus the structures of components **IIIc** and **IIId** were determined to be 2‴-demethoxytylosin and 2‴-demethoxy-4‴-*epi*-tylosin, respectively.

In the ^{1}H NMR spectrum of component **Vb**, on the other hand, the signals of 1‴-H, 2‴-H and 3‴-H were located at 4.52 ppm (d, $J_{1''',2'''}$=7.5 Hz), 3.48 ppm (dd, $J_{2''',3'''}$=3 Hz) and 3.76 ppm (dd, $J_{3''',4'''}$=3 Hz), respectively. Due to the missing 2‴-*O*-methyl, the 2‴-H signal of component **Vb** which corresponds to 3.04 ppm in tylosin[9] shifted downfield to 3.48 ppm, whereas the 3‴-H signal was seen at the same position (3.76 ppm), indicating that *O*-methylation occurred at the C-3‴ hydroxyl group in component **Vb**. The upfield shift of C-2‴ by 9.3 ppm and lack of one *O*-methyl carbon in the ^{13}C NMR spectrum of component **Vb** revealed that component **Vb** had a hydroxyl at C-2‴ instead of *O*-methyl.

The spectroscopic data mentioned above allowed to conclude that component **Vb** was 2‴-*O*-demethyltylosin, which is the position isomer of macrocin (3‴-*O*-demethyltylosin), a biosynthetic intermediate of tylosin[5].

Biosynthetic Considerations

The preceding structure elucidation suggested the possibility that, like macrocin, components **IIIc**, **IIId** and **Vb** might also be biosynthetic precursors for tylosin. Even if they were shunt metabolites, information on the biosynthesis of the third sugar moiety of these macrolides would be helpful for future work on tylosin such as gene engineering.

As reported in a previous paper[4], mutant strain YO-9010 produced component **IIIa** (=demycinosyltylosin) as the major product and components **IIIb**, **IIIc** and **IIId** as minor ones. Because of the limited amounts of components **IIIc** and **IIId** available, no bioconversion studies have yet been done with these components. In consideration of the product composition of mutant YO-9010 observed under the specified fermentation conditions [percent composition of each component: **IIIa** 74%; **IIIb** 5%; **IIIc** 5%; **IIId** 5%; other minor components <2% each: Analyzed by HPLC (columnYMC-Pack A-312; mobile phase 40% acetonitrile in 0.85 M aqueous sodium perchlorate, pH 2.5)], it seems likely that YO-9010 is blocked in the steps of biosynthesis of 6-deoxy-D-allose (a putative precursor for mycinose), as suggested for a similar mutant, GS48, previously reported by BALTZ and SENO[8], assuming that a common sugar precursor is transformed to the third sugar moiety of components **IIIb**, **IIIc** and **IIId**. In light of the pathway of tylosin biosynthesis proposed by BALTZ *et al.*[5], these 2‴-demethoxytylosin isomers are now assumed to be shunt metabolites and not to be convertible to tylosin.

A new mutant numbered TK-22563 produced a large amount of component **Va** (=2‴-*O*-demethylmacrocin) and a lesser amount of component **Vb** (percent compositions of components **Va** and **Vb** analyzed by HPLC: 76 and 13, respectively). Judging from the results of BALTZ and SENO (strain GS16)[8], it seemed interesting to check if component **Vb** was bioconvertible to tylosin by the tylosin producer. For clear differentiation from the endogenous tylosin production, macrolide-non-producing mutants of *S. fradiae* (YT-I and YT-L) were employed in the bioconversion study. The results in Table 4 show that component **Vb**, 2‴-*O*-demethyltylosin, was not methylated at 2‴-OH, while 2‴-*O*-demethylmacrocin was efficiently converted to tylosin, as claimed by BALTZ and SENO[8], and that hydroxylation at C-20 occurred independently of methylation. In other words, once 2‴-*O*-demethylmacrocin is methylated at 3‴-OH, no further methylation occurs, even if 2‴-*O*-methylase is present. It is interesting to note that mutant TK-22563, a producer of components **Va** and **Vb**, efficiently converted

Table 4. Bioconversion of component **Vb** and 2‴-*O*-demethylmacrocin by macrolide-non-producing mutants of *Streptomyces fradiae*.

Mutant	Macrolide	Percent composition			
		Tylosin	Relomycin	2‴-*O*-Demethyl-tylosin	20-Dihydro-2‴-*O*-demethyltylosin
YT-I	2‴-*O*-Demethylmacrocin	85	15	0	0
	Component **Vb***	0	0	93	7
YT-L	2‴-*O*-Demethylmacrocin	68	32	0	0
	Component **Vb***	0	0	78	22
TK-22563	Macrocin	64	25	0	0

* Component **Vb**: 2‴-*O*-Demethyltylosin.

Table 5. *In vitro* antimicrobial spectra of components **IIIc**, **IIId**, **Vb** and tylosin (MIC in μg/ml).

Test microorganism	**IIIc**	**IIId**	**Vb**	Tylosin
Bacillus circulans ATCC 9966	0.10	0.20	1.56	0.20
B. licheniformis	0.20	0.39	3.13	0.39
B. subtilis ATCC 6633	0.10	0.10	0.39	<0.10
Corynebacterium diphtheriae ATCC 11913	<0.10	<0.10	<0.10	<0.10
C. equi IAM 1038	<0.10	1.56	25	3.13
Microbacterium flavum ATCC 10340	<0.10	0.10	0.20	<0.10
Micrococcus luteus ATCC 9341	<0.10	<0.10	<0.10	<0.10
Staphylococcus aureus FDA 209P	0.39	0.39	0.78	0.39
S. aureus Smith	0.39	0.39	0.78	0.39
S. aureus S-29	0.39	0.78	3.13	0.78
S. aureus BX-1633	0.39	0.78	3.13	0.39
S. epidermidis ATCC 14990	0.39	0.39	0.39	0.39
Streptococcus pneumoniae IID 553*	0.39	0.20	0.20	<0.10
S. pyogenes NY 5*	0.20	0.20	0.20	0.39
*S. faecalis**	3.13	3.13	6.25	3.13
*S. viridans**	3.13	3.13	6.25	3.13
Alcaligenes viscolactis ATCC 9036	6.25	1.56	3.13	3.13
Escherichia coli NIHJ	50	50	50	100
Shigella sonnei EW 33	100	50	100	100
Klebsiella pneumoniae ATCC 10031	>100	>100	>100	>100
Proteus vulgaris OXKUS	>100	>100	>100	>100
Pseudomonas aeruginosa IFO 3445	>100	>100	>100	>100
Salmonella gallinarum ATCC 9184	>100	100	100	>100
Serratia marcescens	>100	>100	>100	>100

Determined by the agar dilution method using Mueller-Hinton agar or heart infusion agar supplemented with 10% horse blood (*).

Inoculum size: 5 μl of 10^6 cells/ml.

macrocin to tylosin (the balance of 11% corresponds to the amount of component **Va** endogenously produced during bioconversion). This is consistent with data presented by BALTZ and SENO[3] which indicated that the GS16 mutant cosynthesized tylosin with the mutant (GS15) producing macrocin, and that the GS16 mutant had a at least parental level of the macrocin *O*-methyltransferase. At present, no explanation can be provided for 3‴-*O*-methylation in TK-22563. Thus, component **Vb** is also a shunt metabolite which is not convertible to tylosin.

Comparative *in vitro* Antimicrobial Activities of Components **IIIc**, **IIId**, **Vb** and Tylosin

Table 5 summarizes the comparative *in vitro* antimicrobial spectra of the new analogs and tylosin. In general, the difference in the third sugar moiety of components **IIIc** and **IIId** from tylosin had no effect on the antimicrobial activity. In contrast, the absence of the 2'''-*O*-methyl group in component **Vb** resulted in a significant reduction in antimicrobial potency, as is the case with macrocin. The assay data in Table 5 clearly show that the third sugar moiety of tylosin is very important for expression of the antimicrobial activity. The presence and type of the 4''-*O*-acyl group in tylosin derivatives were described to critically affect the antimicrobial activity against macrolide-resistant pathogens[10]. It is expected therefore that the structure-activity information on the mycinose moiety collected in this paper will be advantageously reflected in future development of tylosin derivatives which are effective *in vitro* and *in vivo* against macrolide-resistant clinical isolates.

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