Communications to the editor

NEW MACROLIDE ANTIBIOTICS PRODUCED BY MUTANTS FROM *STREPTOMYCES FRADIAE* NRRL 2702

Sir:

During the course of a study of microbial transformation of macrolide antibiotics, as reported previously,[1,2] we discovered 4''-*O*-acyltylosins which had great antimicrobial activity against macrolide-resistant Gram-positive bacteria and mycoplasmas than tylosin itself. Therefore, a study was undertaken of macrolides produced by mutants of a tylosin-producing strain. In the present paper, we briefly report the isolation of new macrolide antibiotics which are different from tylosin in the mycinose moiety.

Spore suspensions of *Streptomyces fradiae* NRRL 2702 which produced tylosin were treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. Mature spores of mutant strains which grew on ISP-2 medium were inoculated into 50 ml of culture medium (rape oil 6%, dried yeast 3%, yeast extract 0.1%, K_2HPO_4 0.1%, $CaCO_3$ 0.1%, $MgSO_4 \cdot 7H_2O$ 0.1%) in a 500-ml Erlenmeyer flask and cultured for 6 days at 30°C on a rotary shaker. Macrolides in 1 ml of cultured broth were extracted with 1 ml of ethyl acetate, and 10 μl of the extract was spotted on a TLC plate (silica gel $60F_{254}$ precoated, E. Merck, Darmstadt) and developed by a solvent system of acetone-benzene (2: 1, v/v). The TLC plate was air-dried, dipped in 10% H_2SO_4 and heated at 105°C for 15 minutes. Of about 12,000 colonies examined, 40 mutants lacked the ability to produce tylosin. Among these 40 mutants, the strain YO-10246 produced compound **I** (Rf 0.84); the strain YT-3927 compound **II** (Rf 0.37); the strain YO-9010 compound **IIIa** (Rf 0.23), **IIIb** (Rf 0.31), **IIIc** (Rf 0.26) and **IIId** (Rf 0.24); the strain YO-7625 compound **IVa** (Rf 0.03), **IVb** (Rf 0.23) and **IVc** (Rf 0.17); and other four strains desmycosin; the rest (32 strains) produced no macrolide related compounds.

I (800 mg) was isolated from cultured broth (2.5 liters) of the strain YO-10246 by extraction with toluene followed by silica gel column chromatography with chloroform. **II** (730 mg) was isolated from cultured broth (2.5 liters) of the strain YT-3927 by extraction with ethyl acetate followed by silica gel column chromatography with benzene - acetone (3: 2, v/v). Ethyl acetate extraction of 5 liters of cultured broth of the strain YO-9010 followed by silica gel column chromatography with chloroform - methanol (95: 5, v/v) gave 2,250 mg of **IIIa**, 90 mg of **IIIb**, 110 mg of **IIIc** and 20 mg of **IIId**. **IIIc** and **IIId** were not pure enough for physico-chemical characterization and are under further purification. From ethyl acetate extraction of 5 liters of cultured broth of the strain YO-7625, 2,100 mg of **IVa**, 240 mg of **IVb** and 70 mg of **IVc** were isolated by silica gel column chromatography using a mixture of chloroform - methanol (95: 5, v/v).

The chemical structures of these compounds were determined by spectroscopy and chemical degradation. The IR spectra of these compounds closely resembled each other except for **IIIb** which had a strong absorption band at 1637 cm^{-1}. The physico-chemical properties of **II**, **IIIa**, **IIIb** and **IVa** are shown in Table 1. **I** and **IVc** were identical with protylonolide and macrocin reported by ŌMURA *et al.*[3] and HAMILL *et al.*[4], respectively, by ^1H NMR, ^{13}C NMR and mass spectroscopy.

In the ^1H NMR spectra of **II**, **IIIa**, **IIIb** and **IVa**, the signals of aldehyde, olefin, *N*-dimethyl and C-12 methyl were observed, but the signals of two *O*-methyl groups which were characteristic for mycinose were not recognized. **IIIb** had an *O*-methyl signal. In the spectrum of **II** irradiation at the 2.75 ppm methine (H-14) multiplet led to the collapse of both the methyl doublet at 1.09 ppm and the olefinic doublet at 5.97 ppm to a singlet, indicating that **II** posses a methyl group at C-14. The spectrum of **IIIa** was similar to that of **II** except for the signal at 3.69 ppm instead of 1.09 ppm in **II**, indicating CH_2OH at C-14. The spectrum of **IVa** was more complicated than those of **II** and **IIIa** in the region of 3 ~ 4 ppm, but the methine doublet at 4.52 ppm which was attributable to the anomeric proton of the third sugar was observed, as in the case of tylosin. The spectrum of **IIIb** showed an olefinic doublet at 5.41 ppm (H-3'''), an anomeric doublet of doublets at 5.41 ppm (H-1'''), a

Table 1. Physico-chemical properties of compounds **II**, **IIIa**, **IIIb** and **IVa**.

		II	IIIa	IIIb	IVa
Melting point (°C)		165~168	138~142	127~132	133~137
$[\alpha]_D^{23}$ (MeOH)		−6.6° (*c* 2.0)	−47.4° (*c* 1.0)	−19.3° (*c* 1.1)	−37.4° (*c* 1.5)
λ_{max}^{MeOH}nm($E_{1cm}^{1\%}$)		283 (300)	283 (272)	282 (249)	283 (236)
Molecular weight		725	741	881	887
Formula		$C_{38}H_{63}NO_{12}$	$C_{38}H_{63}NO_{13}$	$C_{45}H_{71}NO_{16}$	$C_{44}H_{73}NO_{17}$
Elemental analysis (%) Found (Calcd.)	C:	63.03 (62.87)	61.36 (61.52)	61.49 (61.27)	59.95 (59.51)
	H:	8.80 (8.75)	8.72 (8.56)	7.95 (8.11)	8.20 (7.95)
	N:	1.84 (1.93)	1.76 (1.89)	1.52 (1.59)	1.51 (1.58)
Rf values (SiO_2 TLC plate)	A:	0.37	0.23	0.31	0.03
	B:	0.58	0.23	0.62	0.04

Solvent system A: C_6H_6 - Me_2CO, 1:2
Solvent system B: $CHCl_3$ - MeOH - 25% aqueous NH_3, 15:1:0.1

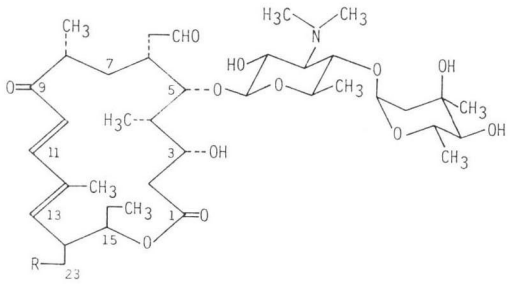
Table 2. ^{13}C NMR chemical shifts for **II**, **IIIa**, **IIIb**, **IVa** and tylosin.

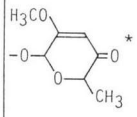
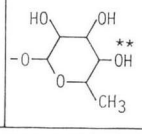
Carbon	II	IIIa	IIIb	IVa	Tylosin	Carbon	II	IIIa	IIIb	IVa	Tylosin
1	174.0	174.0	174.0	174.2	174.2	1′	103.8	103.8	103.8	103.8	104.0
2	39.5	39.5	39.4	39.6	39.5	2′	68.8	68.8	69.0	68.9	69.0
3	71.8	71.8	71.5	71.6	71.9	3′	67.9	68.0	68.0	68.0	68.5
4	38.8	47.2	44.9	45.1	45.2	4′	76.5[a]	76.5[a]	76.5[a]	76.5[a]	76.6[a]
5	81.4	81.4	81.5	81.5	81.5	5′	73.2	73.2	73.1	73.1	73.3
6	32.0	32.1	32.2	32.2	32.1	6′	19.0	19.0	19.0	19.1	19.1
7	32.8	32.8	32.8	32.9	32.9	NMe_2	42.0	42.0	42.0	42.0	42.0
8	40.3	40.3	40.0	40.3	40.4	1″	96.5	96.6	96.3	96.5	96.7
9	203.1	203.5	202.8	203.5	203.2	2″	41.0	41.0	40.9	41.0	41.0
10	118.6	119.1	119.0	118.8	118.9	3″	69.5	69.5	69.6	69.6	69.6
11	148.2	148.1	147.9	148.4	148.4	4″	75.2[a]	75.1[a]	75.0[a]	75.3[a]	75.3[a]
12	133.4	135.9	135.4	135.2	135.1	5″	66.1	66.1	66.3	66.2	66.2
13	146.0	141.8	141.6	142.1	142.5	6″	18.3	18.3	18.3	18.3	18.3
14	44.8	44.6	44.7	44.6	44.8	7″	25.4	25.4	25.5	25.4	25.5
15	78.8[a]	75.1	75.0	74.9	75.3	1‴			97.3	101.2	101.3
16	24.8	25.6	25.5	25.4	25.5	2‴			150.5	71.4[b]	82.1[b]
17	9.1	9.0	9.0	9.0	9.0	3‴			113.1	70.1[b]	80.0[b]
18	9.6	9.7	9.6	9.7	9.7	4‴			192.1	72.9	72.9
19	43.8	43.8	43.8	43.9	43.9	5‴			74.6	71.4	70.7
20	203.0	203.0	203.2	203.1	203.4	6‴			16.9	17.8	17.8
21	17.4	17.3	17.4	17.3	17.4	2‴-OMe			55.2		59.8
22	12.9	13.1	13.1	13.0	13.0	3‴-OMe					61.8
23	16.2	62.4	68.8	69.2	69.2						

^{13}C NMR spectra were recorded at 25.2 MHz in $CDCl_3$. Chemical shifts are given in ppm relative to Me_4Si as internal standard.

a), b) Assignments may be interchanged.

Fig. 1. Chemical structures of compounds **II**, **IIIa(IVb)**, **IIIb** and **IVa**.



Compound	R	Producer
II	-H	YT-3927
IIIa (IVb)	-OH	YO-9010 (YO-7625)
IIIb		YO-9010
IVa		YO-7625

* R: 2-*o*-Methyl-3,6-dideoxy-hex-2-enopyranos-4-ulose

** R: 6-Deoxyallose

methoxy singlet at 3.66 ppm (2‴-OMe) and a methyl doublet at 1.48 ppm (H-6‴), which were all due to the third sugar. Irradiation at 4.19 ppm (H-5‴) converted the doublet (H-6‴) at 1.48 ppm to a singlet, resulting in the clear revelation of the long-range coupling (J=1.8 Hz) between the anomeric proton (H-1‴) and the olefinic proton (H-3‴).

^{13}C NMR data of **II**, **IIIa**, **IIIb** and **IVa** are summarized in Table 2. The proton decoupled spectra showed that both **II** and **IIIa** consist of 38 carbon atoms, and **IIIb** and **IVa** have 45 and 44 carbon atoms, respectively. Compared with the spectrum of tylosin, significant differences were observed at C-23 and the mycinose moiety, while the aglycone, mycaminose and mycarose were the same. The spectrum of **IIIa** showed the lack of mycinose. The signal of C-23 appeared at 62.4 ppm which was 6.8 ppm higher than that of tylosin. This up-field shift indicates the absence of the glycosidic linkage at the C-23 hydroxyl group[5]. The spectra of **IVa** and **IIIb** showed that the third sugars were attached to their C-23 hydroxyl groups. In comparison with the mycinose moiety in tylosin, the up-field shifts of C-2‴ (10.7 ppm) and C-3‴ (9.9 ppm) were observed together with the lack of two *O*-methyl carbons in the third sugar of **IVa**, suggesting the presence of hydroxyl groups at C-2‴ and C-3‴ instead of *O*-methyl groups of tylosin. In the spectrum of **IIIb**, seven carbons were recognized as the constituents of the third sugar and consisted of one anomeric, two ethylenic, one carbonyl, one methine and one methyl carbon. One of the ethylenic carbons (150.5 ppm) showed a singlet and the other (113.1 ppm) a doublet in the off-resonance decoupled spectrum, indicating that the *O*-methyl group was attached to the ethylenic carbon (150.5 ppm). As already described for proton NMR, a long range coupling was shown between protons at C-1‴ and C-3‴, and the *O*-methyl group at C-2‴ was shown. The methoxyenone functionality in the third sugar moiety of **IIIb** was also shown by a strong absorption at 1637 cm^{-1} in the IR spectrum. UV absorption maximum based on the methoxyenone chromophore was not clearly shown, being overlapped with the absorption of dienone chromophore.

From these results, the chemical structure was proposed for the new sugar moiety of **IIIb** as shown in Fig. 1. **IVb** was identified as **IIIa** by all spectral data. **II**, **IIIa (IVb)** and **IVa** were identified to be demycinosyl-23-deoxytylosin, demycinosyltylosin and demethylmacrocin as shown in Fig. 1. Among the compounds described above, **II**, **IIIa** and **IVa** were recently reported by BALTZ and SENO[6], although the data for their structural determination were not described.

The chemical structures of **IIIc** and **IIId** and the configuration of the third sugar in **IIIb** will be reported in the near future.

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