BIOLOGICAL GLYCOSIDATION OF MACROLIDE AGLYCONES

I. ISOLATION AND CHARACTERIZATION OF 5-O-MYCAMINOSYL NARBONOLIDE AND 9-DIHYDRO-5-O-MYCAMINOSYL NARBONOLIDE

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Glycosidation of narbonolide with mycaminose was attempted by feeding narbonolide during the fermentation of a parent or a mutant strain of *Streptomyces platensis*, a producer of 16-membered macrolide antibiotics, platenomycins. As a result, two new compounds **I** and **II** were isolated from the fermentation broth and identified as 5-O-mycaminosyl narbonolide (**I**) and 9-dihydro-5-O-mycaminosyl narbonolide (**II**), respectively. Physicochemical and antimicrobial properties of **I** and **II** are also referred to.

Hitherto, a number of aglycones of macrolide antibiotics have been isolated,[1~7] and used for the elucidation of the biosynthetic pathway of this antibiotic group, *e.g.* erythromycin,[8] picromycin[9] and platenomycins.[10]

As reported previously, the authors isolated narbonolide, the aglycone of narbomycin, from the culture of *Streptomyces venezuelae* MCRL-0376 which produced narbomycin and picromycin as major antibiotics,[11,12] and succeeded in the bioconversion of biologically inactive narbonolide into active antibiotics (narbomycin and picromycin) by glycosidation of the aglycone.[9]

In 1972, rosamicin was reported[13] to consist of an aglycone identical with that of cirramycin A_1 and a sugar which was identified as desosamine. Considering that rosamicin is constructed from a 16-membered aglycone and a sugar usually found in 12- or 14-membered macrolide antibiotics, it was conversely assumed that a new antibiotic might be obtained by combining a sugar normally present in 16-membered macrolide antibiotics with a 14-membered macrolide aglycone.

Thus, glycosidation of narbonolide with mycaminose (a sugar component of 16-membered macrolide antibiotics, such as platenomycin, leucomycin, spiramycin, cirramycin, tylosin, carbomycin *etc.*) was attempted by adding narbonolide during the fermentation of platenomycin-producing *Streptomyces platensis* MCRL-0388[14] and its blocked mutant (strain U-21). Two new compounds **I** and **II** were successfully derived from narbonolide.

In this paper, the isolation and characterization of these biotransformed compounds **I** and **II** as 5-O-mycaminosyl narbonolide and 9-dihydro-5-O-mycaminosyl narbonolide respectively is described.

Microorganism and Fermentation

The strains employed in this investigation were *Streptomyces platensis* subsp. *malvinus* MCRL-0388, a producer of the platenomycins and a mutant strain U-21. Strain U-21 was derived by treatment of the parent strain with ultraviolet irradiation. Strain U-21 was completly blocked in an early stage of platenomycin biosynthesis and unable to synthesize the antibiotics *de novo*. However, this

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mutant can synthesize platenomycins in the presence of certain intermediates such as platenolides I and II.[10]

The fermentations of these organisms were carried out as follows: seed cultures were obtained by incubating the spores at 27°C for 62 hours on a rotary shaker (180 rpm) in 100 ml of medium consisting of glucose 20 g, meat extract 7.5 g, yeast extract 3 g and NaCl 3 g per liter (tap water, pH unadjusted) prepared in 500 ml Erlenmeyer flasks. Then, the seed culture was inoculated at a level of 2% (v/v) into 500 ml Erlenmeyer flasks containing 100 ml of fermentation medium consisting of corn starch 15 g, soy bean meal 15 g, cornsteep liquor 1 g, yeast extract 2 g, NaCl 5 g and $CaCO_3$ 2 g per liter (tap water, pH unadjusted), and incubated at 27°C on a rotary shaker (180 rpm).

Narbonolide dissolved in ethyl alcohol was added to a 20-hour culture of the organisms at the concentrations indicated. The fermentation was further continued and analyzed for products at 20, 40, 70 and 90 hours after addition. Antibiotic activity in the broth was assayed at the times indicated by a cup plate method using *Bacillus subtilis* PCI-219 as a test organism.

Production of the Biotransformed Compounds I and II

The production of platenomycins by MCRL-0388 after addition of narbonolide was first examined. Fig. 1 shows the result. When narbonolide was added, platenomycin production was depressed, the depression being more marked as the narbonolide concentration became higher. At the levels of 200 mcg/ml of narbonolide, only 20 mcg/ml of platenomycins was produced. The production of platenomycins was depressed to about 5% of the control value.

Since it was expected that the organism may accumulate some narbonolide, the fermentation broth was examined by thin-layer (TL) chromatography on silicagel G (Merck) with a solvent system of chloroform - methanol - acetic acid - water (79: 11: 8: 2). The TL chromatogram of an ethyl acetate extract of the beer at 40, 70 and 90 hours after addition of narbonolide indicated the presence of two new products, tentatively designated as **I** and **II**, which increased as the added narbonolide disappeared. These compounds could be distinguished from platenomycins by their color on TL-plates (colorlation after spraying with H_2SO_4 followed by heating: platenomycins, reddish violet or blue; **I** and **II**, light

Fig. 1. Effects of narbonolide on the production of platenomycins in *Streptomyces platensis* MCRL-0388.
Narbonolide was added at 20 hours.

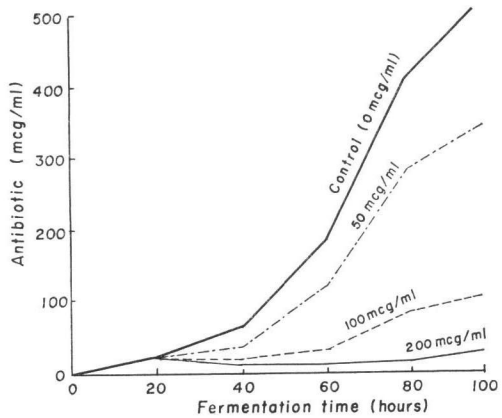
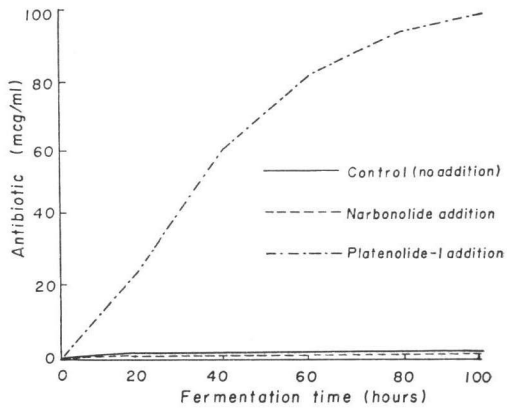


Fig. 2. Effects of platenolide and narbonolide on the production of platenomycins in strain U-21.
Platenolide-I or narbonolide was added at time 0 at the concentration of 100 mcg/ml.



brown resembling narbonolide.).

The isolation of the two new products was attempted using the fermentation beers of MCRL-0388 at an earlier stage, but many difficulties arose in the purification process because of the similar solubility of the new compounds and the platenomycins. Therefore, experiments were conducted using a mutant strain, U-21 which is unable to synthesize platenomycins. Fig. 2 shows the result of the fermentation added with platenolide I or narbonolide. Antibiotic activity was observed only when platenolide I was added to the medium, and the active products were identified as platenomycins by TL-chromatography. In contrast, the fermentation added with narbonolide gave two new compounds. They were identical with the products noticed during the fermentation of the parent strain.

Isolation and Purification of Compounds I and II

The fermentation beer (10 liters) of strain U-21 was filtered through Celite 545, and the filtrate was extracted with ethyl acetate at pH 8.0. After concentrating the extract, biotransformed products in the solvent were transferred into diluted hydrochloric acid at pH 3.0. The acidic solution was adjusted to pH 8.0 with 1 M NaOH and extracted with benzene. The benzene layer was concentrated to dryness to yield a crude powder (36.7 g), which was then dissolved in ethyl acetate and chromatographed on a silica gel column using a solvent system of chloroform - methanol (9: 1). The mixture of the products obtained as crude powder by the above chromatography was separated into pure components on an alumina (Woelm, neutral, activity III) column. Compound **I** was eluted by an ethyl acetate -benzene (9: 1) mixture, and **II** was then eluted by ethyl acetate. Each eluate was monitered by TL chromatography visualised with 40% H_2SO_4 spraying followed by heating. Each compound thus obtained was recrystallized from chloroform - *n*-hexane as colorless needles. Thus 21 mg of **I** and 128 mg of **II** were obtained.

Physicochemical Properties and Structure of Compounds I and II

The compounds **I** and **II** finally elucidated as 5-O-mycaminosyl narbonolide and 9-dihydro-5-O-mycaminosyl narbonolide respectively are soluble in methanol, ethanol, acetone, ethyl acetate, chloroform and benzene, and less soluble in *n*-hexane and petroleum ether. They gave negative erythromycin and carbomycin tests, and negative FEHLING, ferric chloride and ninhydrin tests. On the TL chromatogram these compounds were visualized as light brown spots by spraying 40% H_2SO_4 followed by heating. Rf values and other physicochemical properties were listed in Tables 1 and 2.

IR spectrum of **I** suggested the presence of hydroxyl (3450 cm^{-1}), lactone (1740 cm^{-1}), conjugated ketone (1695 cm^{-1}), simple ketone (1705 cm^{-1}) functions together with ethylene linkage (1630 cm^{-1}). UV absorption at 225 nm (log. ε 4.08) in EtOH showed the presence of an α, β unsaturated ketone, and the maximum at 288 nm in alkaline EtOH suggested the presence of a β-keto-lactone system[15] in **I**.

The ^{1}H-NMR spectrum of **I** measured in $CDCl_3$ indicated the presence of seven C-methyl (δ 0.8~1.5, 21 H), one N-dimethyl (δ 2.60, s, 6 H) and two olefinic (δ 6.06, 1 H, d, J=16 and δ 6.67, 1 H, dd, J=16, 6) protons.

The mass spectrum of **I** showed a molecular ion peak at *m/e* 525 and intense fragment peaks at *m/e* 351, 335, 190 and 174. By total acetylation, **I** gave a diacetate, whose mass spectrum showed peaks at *m/e* 609 (M^+), 351, 335, 274 and 258.

These data indicate that compound **I** is closely related to narbomycin. However, judging from its

Table 1. Physicochemical properties of compounds **I** and **II**

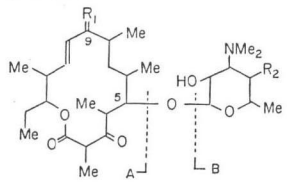
	Compound **I**	Compound **II**	Narbomycin
Appearance	Colorless needles	Colorless needles	Colorless needles
m.p. (°C)	85~87	94~97	113~115
Formula	$C_{28}H_{47}NO_8$	$C_{28}H_{49}NO_8$	$C_{28}H_{47}NO_7$
Mol. Wt. (MS)	525 (M^+)	527 (M^+)	509 (M^+)
$UV\lambda_{max}^{EtOH}$ (nm) (log. ε)	225 (4.08)	End absorption	224 (4.08)
$UV\lambda_{max}^{EtOH+NaOH}$ (nm)	288	288	288
IR^{nujol} cm^{-1}	3450, 1740, 1705, 1695, 1630, 1460, 1380, 1330, 1265, 1195, 1170, 1080, 1060, 800	3450, 1740, 1705, 1460, 1375, 1325, 1265, 1190, 1165, 1080, 1060, 835	3450, 1740, 1705, 1695, 1670, 1630, 1460, 1380, 1350, 1280, 1220, 1190, 1150, 1110, 1070, 1040, 980
Rf value*	0.57	0.45	0.65

* TLC on silica gel G (Merck) plate.
Solvent system: $CHCl_3$ – MeOH – AcOH – H_2O (79: 11: 8: 2).

Table 2. Diagnostic fragment peaks of compounds **I**, **II** and narbomycin in mass spectra

	Compound **I**	Compound **II**	Narbomycin
M^+	*m/e* 525 (*m/e* 609)*	*m/e* 527 (*m/e* 653)	*m/e* 509 (*m/e* 551)
A	*m/e* 335 (*m/e* 335)	*m/e* 337 (*m/e* 379)	*m/e* 335 (*m/e* 335)
B	*m/e* 174 (*m/e* 258)	*m/e* 174 (*m/e* 258)	*m/e* 158 (*m/e* 200)

* ( ): acetyl derivative



	R_1	R_2
Compound I	=O	-OH
Compound II	-OH,H	-OH
Narbomycin	=O	-H

lower Rf value in TL chromatography, **I** was thought to be more polar in nature than narbomycin. IR, UV and ^{1}H-NMR spectra of **I** and narbomycin were hardly distinguishable. However, the mass spectra of **I** and narbomycin showed the 16 mass unit difference in the molecular ion (*m/e* 525 and 509 respectively). The intense fragment peaks of compound **I** at *m/e* 351 and 335 were identical with those of narbomycin and ascribed to an aglycone part. The intense peaks at *m/e* 190 and 174 were comparable respectively to *m/e* 174 and 158 of narbomycin. The latter two were due to a sugar moiety of narbomycin. The peaks at *m/e* 190 and 174 shifted to *m/e* 274 and 258 with a peracetyl derivative, the shifts corresponding to two acetyl units. By this comparative mass spectral analysis, the presence of mycaminose which has one more hydroxyl function than desosamine was assumed in **I**, and **I** was presumed to be 5-O-mycaminosyl narbonolide, in which the desosamine moiety in narbomycin is replaced by mycaminose.

Hydrolysis of compound **I** with 6 N hydrochloric acid gave an amino sugar, which was detected to be D-mycaminose by direct comparison with an authentic sample obtained from platenomycins.

The structure of compound **II** was readily suggested, when IR, UV and ^{1}H-NMR spectra of **II**

were analyzed in comparison with those of **I**. The mass spectrum of **II** showed intense ion peaks at *m/e* 527 (M^+), 353, 337, 190 and 174, and on its totally acetylated compound, the spectrum showed intense ion peaks at *m/e* 653 (M^+), 395, 379, 274 and 258. Therefore, **II** was estimated to have three hydroxy groups which can be acetylated and have two more hydrogens than **I**. The ion peaks of **II** at *m/e* 190 and 174, ion peaks of the triacetate at *m/e* 274 and 258, and signals in ^{1}H-NMR assigned as seven C-methyls (δ 0.8~1.25, 21 H) and one N-dimethyl (δ 2.57, s, 6 H) were the same as those in **I**. Consequently, **II** was assumed to possess a mycaminose moiety as with **I**. Fragment ion peaks of **II** at *m/e* 353 and 337 may be comparable to the peaks of **I** at *m/e* 351 and 335 respectively. These peaks were ascribed to an aglycone part. However, contrary to the peaks of **I** which were observed unchanged in its diacetate, the peaks of **II** shifted to *m/e* 395 and 379 respectively on its triacetate, the shift corresponding to one acetyl unit. Therefore, an aglycone of **II** may have one more hydroxyl group than **I**. Considering this assumption together with the fact that **II** has two more hydrogens than **I** and lacks the α,β-conjugated ketone system, the aglycone of **II** was supposed to be a dihydroaglycone of **I**. Thus, **II** was assumed to be a dihydro-**I**, namely, 9-dihydro-5-O-mycaminosyl narbonolide.

The above assumption was chemically confirmed by mild oxidation of **II** to **I**. Oxidation of **II** with active MnO_2 in $CHCl_3$ gave a dehydro derivative, whose IR, UV, Mass and ^{1}H-NMR spectra were completely identical with **I**.

Biological Activity of Compounds I and II

The antimicrobial activity of compounds **I** and **II** were determined by an agar dilution method. The results are shown in Table 3. Compound **I** showed antibacterial activity against Gram-positive bacteria, but the activity was considerably less than that of narbomycin. On the other hand, compound **II** showed almost no antimicrobial activity.

Table 3. Antimicrobial activity of compounds **I** and **II**

Test organism	MIC (mcg/ml)		
	Compound **I**	Compound **II**	Narbomycin
Staphylococcus aureus FDA 209 P	1.56	>100	0.39
S. aureus Terashima	1.56	>100	0.39
S. aureus Smith	1.56	>100	0.39
S. epidermidis 10131	1.56	>100	0.39
Streptococcus faecalis	1.56	>100	0.39
Escherichia coli NIHJ	>100	>100	>100
E. coli K-12	>100	>100	>100
E. coli FE-216	>100	>100	>100
Salmonella typhimurium	>100	>100	>100
Klebsiella pneumoniae	>100	>100	>100
Pseudomonas aeruginosa A_3	>100	>100	>100
Proteus vulgaris	>100	>100	>100

Discussion

Since the isolation of picromycin by BROCKMANN and HENKEL, more than thirty macrolide antibiotics have been isolated. These antibiotics possess a large ringed lactonic aglycone and one or more sugars. The lactone part and the sugars are not active by themselves alone, and their combination is

required for antibiotic activity. A difference in the sugar moiety linked to the same aglycone causes changes in the properties and activity as seen in cirramycin A_1 *vs.* rosamicin. Thus, it should be possible to find new macrolide antibiotics by changing a sugar moiety attached to an aglycone.

In this paper, a new 14-membered macrolide antibiotic is reported which possesses mycaminose, hitherto recognized only as a sugar present in 16-membered macrolide antibiotics. Further, it should be possible to prepare a rosamicin-like antibiotic in which mycaminose was replaced by desosamine, an amino sugar normally found to bound to 12 and/or 14-membered aglycones. This possibility will be dealt with in part II of this series.

As investigated by many scientists, macrolide aglycones were of great use in elucidating the biosynthetic pathway of this antibiotic group by using the aglycones as substrates or precursors in fermentation studies. By this technique, the biosynthetic pathway of narbomycin,[9] picromycin[9] and platenomycins[10] were clearly elucidated in this laboratory.

The present experiment extended the use of macrolide aglycones as a starting material for the preparation of new macrolide antibiotics. However, some problems were left unsolved. For example, no biosynthesis of a macrolide with a disaccharide moiety was observed. In the present experiments, a compound posessing the disaccharide mycarosyl mycaminose was searched for, but the desired compound was not found.

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